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No. 7

AN EXPERIMENTAL ANALYSIS OF THE ORIGIN OF BLOOD AND VASCULAR ENDOTHELIUM

I. THE ORIGIN OF BLOOD AND VASCULAR ENDOTHELIUM IN EMBRYOS WITHOUT A CIRCULATION OF THE BLOOD AND IN THE NORMAL EMBRYO

FORTY-NINE FIGURES

II. A STUDY OF WANDERING MESENCHYMAL CELLS ON THE LIVING YOLK-SAC AND THEIR DEVELOPMENTAL PRODUCTS: CHROMATOPHORES, VASCULAR ENDOTHELIUM AND BLOOD CELLS

THIRTY-FIVE FIGURES

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CONTENTS

INTRODUCTION

The origin of blood presents an almost unique problem in embryology. First, on account of the fact that the initial blood anlage in many animals is contributed to by wandering cells. Second, owing to the establishment of an early flow or circulation of embryonic fluids before the blood corpuscles have arisen.

Soon after the cells and corpuscles are formed they are swept into this circulating current and carried to all parts of the body. In this way the blood cells become associated and mixed with numerous other types of cells, and it is difficult, if not impossible, to establish their true relationship with their surroundings. For the above reasons one is often ready to believe that many of the even careful and long thought out contributions to the development of blood are, after all, largely a matter of the author's own interpretation rather than a record of the actual processes.

The general current of opinion at the present time would seem to indicate that all blood cells arise from a mesenchymal type of cell. A number of very competent workers have described the change of this mesenchymal cell into a stem cell or mother cell. On one side from this mother cell are developed various leucocytes, which it is important to note always occur in an interstitial position, while on the other hand, this same general type of mother cell gives rise to other cells which later differentiate into typical erythroblasts, and finally erythrocytes which are always found to be located within the vessels. These so-called indifferent mesenchymal cells probably, from the evidence contained in the literature, do form blood cells, but to the discriminating reader the evidence is not at all convincing that both white blood cells and red blood cells really arise from one common mother cell or common embryonic anlage.

The possibility, and even probability, is certainly present that these so-called stem mother cells may in reality not all belong to one type, but are different and may already be destined to form either red cells or white cells. Yet on account of their wandering capacities as well as on account of the fact that the earliest blood cells are swept around in the circulating current

they have become so mixed and confused that it is almost impossible to separate the cell groups or differentiate between them. One must in this connection, remember the fact that almost all authors have concluded that only red blood cells are formed in the blood islands of the yolk-sac in most vertebrates. All authors who have studied the development of the blood in Teleosts have invariably described only red blood cells as arising in the intermediate cell mass. No one has ever mentioned the presence of white blood cells in the true blood forming anlage of the Teleosts.

The monophyletic school really goes still further and not only claims that all types of blood cells arise from a common mother mesenchymal cell, but also that the vascular endothelial cell is likewise capable of giving rise to the various types of blood cells and is originally a cell of the same type as the stem mother cell. There are numerous descriptions and illustrations of the origin of blood cells from the vessel linings in the literature of the past twenty-five years, since Schmidt in 1892 described the transformation of individual endothelial cells into white and red blood corpuscles. Yet again, I believe that the really skeptical reader will not be at all convinced that such a thing ever takes place from the evidence presented in the literature, certainly not from any of the illustrations that have been made of this process.

No real vascular endothelial cell has been actually observed to metamorphose into a blood cell or to divide off another cell which forms a blood cell, and until such a direct observation is forthcoming one can only question the accuracy of the interpretation of the various observations up to now recorded.

The mesenchyme is a very generalized embryonic tissue and from it arise the various kinds of blood cells, endothelial cells, connective tissue cells, etc. There can be no doubt of the great genetic difference between blood cells and connective tissue cells, yet their parent cells are with our present methods indistinguishable. We may with equal justification go further and hold likewise that the cells from which the vascular endothelium, red blood cells and white blood cells arise are mesenchymal

cells really differing in nature according to whether they will give rise to one or the other of the three cell types. Yet they may not differ from one another in any way by which we can at present distinguish them. If this proposition be true, or even if the weight of evidence lean in this direction, it is scarcely more justifiable to derive these completely different cells from a common mother cell than it would be to derive connective tissue and blood cells from a common mother cell.

Of course, we are only considering the mesenchymal cell just before its differentiation is to begin. Carried back further, no doubt, all the cells become more and more alike and possess more and more complex potentialities as is so thoroughly demonstrated by the numerous studies of cell lineage. In the beginning, of course, all cells arose from one single egg cell capable of giving rise to every tissue of the body, but after tendencies in differentiation have proceeded sufficiently far in the various cells some then form real mesenchymal cells. Later individual mesenchymal cells incline in certain directions and finally become incapable of giving rise to any other than the definite type of tissue or cells towards which their particular tendencies have directed just as certain endodermal cells become specialized to form the liver while others near by and at first indistinguishable from these give rise to the ducts and acini of the pancreas.

All of the vertebrate classes present these many questions of blood origin, etc., but the forms upon which this investigation has been conducted, the Teleosts, possess in addition many extremely interesting special problems. In all other meroblastic embryos the majority of the earliest blood cells arise in yolk-sac blood islands. Yet in many of the Teleosts there are apparently no early blood islands on the yolk, but all of the blood forming cells are contained within the embryonic body.

This intra-embryonal blood anlage has been frequently described by many authors as the "intermediate cell mass." The intermediate cell mass as has been suggested by Marcus ('05), Mollier ('06), and others, is really the homologue of the blood forming yolk-sac mesoderm in the other meroblastic types.

The bony fish is important as an object of study on account of the fact that so many of its organs and tissues arise in a way peculiar to the group and differing from the other vertebrate classes. The solid gastrular invagination described by Sumner ('00), the original solid condition of the central nervous system, the solid optic knob which changes into the optic vesicle, and in the present connection, the very particularly interesting solid cord of cells, the intermediate cell mass, which is to give rise to the red blood corpuscles of the individual make the Teleosts a group of great embryological interest.

The complexity of the problem concerning the origin of the various types of blood cells is then largely due to the migration and mixture of the cells involved. It is strange that up to now no investigator has attempted in an experimental way to analyze the situation. It would seem to be one of the most favorable problems for an experimental analysis, and in the end it is certainly an analytical problem.

If it were possible by any means to separate the anlage of the red blood cells from that of the white blood cells and prevent the flow of fluid in the embryonic body so that these cells would not frequently become intermixed, then it would seem possible to determine clearly the entire genesis of the various type cells. If all the types of blood corpuscles did arise from a common mother mesenchymal cell they should then be found in intimate association throughout all blood forming regions. Further, if the vascular endothelium really has blood forming power, it should be found that blood cells arise in any region of the embryo which possesses vessels lined by such endothelium.

There have been various experiments performed which have interfered more or less with the circulation of the body fluids of the embryo, but none of these experiments where aimed at a solution of the genesis of blood cells or have been used for such a purpose. Knower ('07) removed the heart anlage from early frog embryos and they continued to develop in some cases with almost no circulation. In other specimens there was a very feeble sluggish circulation due to the pulsation of the lymph

hearts or of remnants of the heart which remained after the operation. The embryos were not particularly adapted for the study of the blood questions since some circulation always took place, and this no doubt was sufficient to contaminate the original sources of blood cells and so confuse the situation. Loeb ('12) has reported experiments on bony fish hybrids and embryos treated with certain chemicals in which there was a heart beat but no circulation. These embryos were, however, not studied for either blood or vascular genesis.

The first demonstration of the fact that the embryo could develop without the circulation of the blood was given by Loeb in 1893. He showed that Fundulus eggs developing in solutions of KCl had no heart beat and no circulation of the blood, yet some vessels formed. In 1906 the writer repeated this experiment and confirmed Loeb's results entirely, but found that the vascular system and general development of the embryo was extremely abnormal and was hardly reliable for conclusive studies on the origin of special tissues.

With these experiments in mind, and appreciating the problems indicated above regarding the origin of blood as well as vascular endothelium, I have undertaken an extensive experimental analysis of this subject in conjunction with a careful systematic study of the histogenesis of the blood and vessels in normal embryos. The results of the experimental study which has been carefully followed during the past three years are presented in the following pages of this paper.

METHODS OF EXPERIMENT AND MATERIAL

Six years ago, while studying the influence of alcohol and various anaesthetics on the development of Fundalus embryos, I noticed that many of these embryos had a feeble heart beat, but no circulation of the blood. At that time, particular attention was given to a study of the defects of the central nervous system and of the organs of special sense and no attempt was made to investigate thoroughly the conditions present in other tissues and organs of the body.

During the last few years, special attention has been devoted to the study of these embryos without a circulation of their body fluids with the main object of analyzing as completely as possible, the origin and subsequent development of the heart, vessels and blood. All of the experiments have been repeated through three summers in the Marine Biological Laboratory at Woods Hole. It has been possible to produce embryos that were almost normal in all particulars yet in which the blood failed to circulate on account of the fact that the heart was blind at one or both ends or disconnected at the venous end or finally was a completely solid cord of tissue.

The embryos were studied in life and the particular individuals which had been so observed were fixed and finally studied in microscopic sections. The observations made on the experimental embryos have in all instances been completely checked and controlled by careful detailed study of the blood and vessels in normal embryos.

The experiments have been performed on two species of Fundulus, heteroclitus and majalis, and the results are practically identical for both. The eggs were stripped from the female into small dishes containing no water and fertilized by milt pressed from the male. About fifteen minutes after the application of the sperm water was added to the eggs. In this way one gets a very high percentage of fertilized eggs, while fertilizing the eggs under water or adding sperm to a dish of water gives a much poorer result. The fertilized eggs are then divided into groups so that the experiment and control are all from similar sources. The eggs just before dividing into the two-cell stage were introduced into solutions of alcohol.

The solutions which gave the most favorable results were prepared in the following way: 50 cc. of sea-water was placed in each dish and to this was added respectively, 1.5 cc., 2 cc. 2.3 cc., 2.6 cc., 2.8 cc., 3 cc., 3.5 cc., of 95 per cent commercial alcohol. The eggs remained in these solutions for twenty-four hours, after which time the solution was renewed. After forty-eight hours the eggs were removed from the alcohol solutions and placed in pure sea-water.

To give a general idea of the way in which the embryos developed in these different grade solutions of alcohol, I may cite some of the details of one experiment. After forty-eight hours many eggs are dead in all the solutions. The dead eggs are thrown out. When seventy-two hours old many others are dead in the solutions with 1.5 cc., 2 cc., 2.3 cc., and 2.6 cc., while all but one individual had died in 2.8 cc. It should be added here that about seventy-five eggs are placed in each dish. From the 1.5 cc. solution sixteen are alive at seventy-two hours, several with various eye defects, the hearts are beating but contain colorless fluid and the only trace of red blood color is in the intermediate cell mass and caudal vein. Several others of this lot have a full circulation with corpuscles in the current. From the 2 cc. solution one has a feeble heart beat but no blood is visible and there is no circulation; some embryos have no circulation but blood is present in the posterior end of the body, while many have blood circulating with a strong heart beat.

This brief reference to the notes of the experiment show that such doses are just on the border line of effectiveness since some individuals in the solutions are not able to develop a circulation, while others with a higher degree of resistence do develop a more or less normal circulation. It is very important in such experiments to use these threshold doses since they are the least injurious possible to give the desired result. In this way one gets individuals which have no circulation of the blood, and, therefore, in which the blood anlage develops and remains in its permanent position, without having any serious defects or abnormalities in the general body tissues of the embryo.

From a study of such specimens carefully controlled by a study of normal individuals, one is fully justified, I believe, in drawing final conclusions as to the significance of the developmental processes taking place. It cannot be argued so far as the blood anlage is concerned that the conditions recorded are pathological or other than those which would occur in a normal genesis of the blood except that it never circulates.

Embryos which are intended for microscopic study have been prepared in the following manner: The eggs are placed

in picro-acetic (saturated aqueous solution of picric acid and 5 per cent glacial acetic) from thirty to forty minutes, then put into 70 per cent alcohol. This is frequently changed in order to wash out the picric acid. After they have been about one-half hour in the 70 per cent alcohol, the egg membrane is removed with fine dissecting needles. This is the most favorable time for removing the membrane. If the eggs have been left for a long time in the alcohol, the membrane is more difficult to remove and the embryo is brittle and more liable to injury.

After removing the entire membrane, the yolk-sac is then punctured at its ventral pole and the yolk mass very slowly and cautiously removed from the sac. To remove the yolk mass, requires a great deal of practice and extreme care in every case. It should be done with the use of a binocular microscope so that the operator can be certain not to tear or destroy the yolk-sac or injure the delicate heart lying close above the yolk. After a great deal of practice it is possible to remove the yolk from a number of embryos and leave the yolk-sac in perfect condition with the heart and pericardium practically undisturbed. In the great majority of cases, however, it is generally impossible to completely remove the yolk. It is usually necessary to remove the yolk on account of the fact that when the eggs are imbedded in either paraffin or celloidin, the yolk becomes so hard that it often breaks the sections and makes it very difficult to get a complete or perfect series. When the yolk-sac is punctured one-half hour after having been in the 70 per cent alcohol, the yolk material is in a gummy or viscid condition and is more easily removed than at any other period tried.

After having removed the egg membrane and the yolk, the embryos are then allowed to stand twenty-four hours in 70 per cent alcohol when they are changed to 80 per cent to be kept until the time of imbedding. The embryos are imbedded in paraffin and cut in serial sections from five to ten micra thick. They are stained in hematoxylin and eosin and extracted or carefully differentiated so as to bring out a clear stain of the blood cells and tissues. A complete series of these embryos have been made from a time before the appearance of blood up to

sixteen days old, the normal embryo hatches and becomes free swimming at about twelve days.

As mentioned above, a similar series of normal embryos have been prepared and used for comparison with these non-circulating individuals.

In order to be certain of the final developmental product of the blood cells of the fish, numerous smears have been made from various tissues and from heart blood taken from the adult Fundulus. In these smears one finds the various types of white blood cells and the ordinary red blood corpuscles of Teleosts.

THE STUDY OF LIVING EMBRYOS WITH AND WITHOUT THE CIRCULATION OF THE BLOOD

1. Normal development up to the establishment of a circulation

The rate of development of Fundulus embryos is very variable, differing at different periods of the breeding season, and also differing in groups of eggs from different individuals.

When twenty-four hours old, the germ ring has descended almost to the equator in the most rapidly developing individuals. In others the ring is only one-third way over the yolk sphere. The embryonic shield and the first line indicating the position of the embryo's body is now to be made out. At forty-eight hours, the yolk sphere is completely covered by the ectoderm, the embryonic body is well shown with the optic knobs projecting prominently and several somites easily distinguishable. The heart has not begun to pulsate and no blood cells or blood anlage are distinguishable in the living specimen. Very soon after this time, or at least by sixty-eight hours, there are about ten somites present and collections of cells on the yolk-sac are the first indication of blood islands. No pigment cells have formed up to sixty hours.

At seventy-one hours pigment cells are recognizable but the blood islands are not yet colored and are sparsely arranged over all the yolk region except the anterior half. Near the lateral borders of the embryo and on the posterior yolk surface the islands are most abundant. At this time there is still no visible

heart-beat or circulation to be seen with the high power microscope. At seventy-five hours the pigment particles are just beginning to show in the chromatophores. A well formed vesicle is clearly seen at the posterior end of the embryo from forty-eight to seventy-two hours and older. This is the so-called Kupffer's vesicle, and it, like the pericardium, becomes greatly distended by an accumulation of plasma in those individuals which have no circulation.

Embryos with fourteen somites may still have no heart beat and no circulation of the blood. Any later than this, however, all normal embryos establish a heart-beat and a circulation of colorless fluid, there being no blood cells present in the initial circulating medium. Very soon after the circulation is established at first a few but quickly many blood cells are added to the stream. This is merely an abbreviated summary of the development of the Fundulus embryo up to the time of the establishment of the heart-beat and circulation, but as stated above, the rate is variable and it often happens that an embryo of seventy-two hours has already established a vigorous circulation and the plasma is loaded with well formed blood cells.

2. History of experimental embryos to the time when a circulation should begin

In the experimental embryos development proceeds more slowly than in the normal. The plasma which should circulate in the vessels accumulates in the sinuses over the yolk and finally seems to collect in great amount in the pericardium, the lateral coelomic cavities and in Kupffer's vesicle, so that these spaces become hugely distended and appear as great sacs or vesicles of colorless watery fluid. The excessive presence of this fluid in the pericardium seems to exert a mechanical effect which tends to separate the head of the embryo an unusually great distance from the yolk mass and thus stretches the heart out into a long string-like cord, passing from the embryo to the surface of the yolk.

This pushing away of the head from the yolk is very well indicated in figures 15 to 20, which show various types of hearts found in these embryos during later stages. The stretching or

pulling out of the heart may possibly be the cause of the failure to develop its proper connections with the veins, or in some cases to establish and maintain its lumen. On account of these mechanical deficiencies in the heart, we find that it is incapable of propelling the body fluids and establishing the circulation of the blood. The fluids thus accumulate in the large sinuses or spaces, in most cases the coelomic spaces and in the case of Kupffer's vesicle in an endodermic or endo-mesodermic cavity.

The red blood cells become evident after the fluid accumulation has partially taken place. They are always seen to originate in definite localities and are never found in any place very distantly removed from these localized regions unless a partial circulation or accident of some kind has occurred. Although red blood cells in many places arise from wandering cells the blood cells themselves have little capcity to wander.

3. Early formation of blood cells in living embryos

a. Intra-embryonic blood cells: The chief place of blood cell formation is the intermediate cell mass which extends from about the level of the anterior portion of the kidney back posteriorly to behind the anus and well into the tail of the embryo. This is the principal blood mass, but in addition to this, there are present in all of these non-circulating individuals small blood islands over the posterior and ventral yolk regions. These blood islands are also present on the yolk of normal individuals but in these the islands are swept away when the circulation begins.

b. Yolk-sac blood islands. A number of observations were made on the embryos which failed to develop a circulation and also on normal embryos to determine the significance and relationships of the blood islands on the yolk as far as was possible in the living individuals.

In one experiment when the embryos were seventy-two hours old, or just about the time that the heart-beat was beginning in many, it was found that although the plasma was circulating blood islands were present on the posterior yolk region. Ten embryos were selected which showed these posterior yolk blood

islands and isolated to determine whether the blood islands would subsequently enter the circulation or what their fate would be. Ten other embryos with a feeble heart-beat but with no circulation yet also showing small posterior yolk-sac blood islands were isolated for comparison.

On the following day, nine out of ten individuals which had established a vigorous circulation had no blood islands remaining on the yolk. All of the islands had been taken into the circulation or vascularized, so that instead of blood islands there was now a network of vessels over that portion of the yolk and the blood cells had entered the current. One of the ten embryos exhibited an abnormal arrangement of the blood vessels that was particularly instructive. On one side there was a large vein running from the embryo out onto the yolk and on this side all of the blood islands had disappeared forming a network of vessels which conducted the blood to the venous end of the heart. On the other side of the specimen there seemed to be a suppression in the development of vessels near the embryo. The islands were still in the same condition they had been on the previous day except that the cells composing them had become much redder so that there was now no doubt whatever that they contained erythroblasts and early erythrocytes.

The ten embryos which had no circulation on the previous day were now found to present the following conditions: Two had established a perfectly free circulation and all of the blood islands had been swept away. In two other individuals circulations were established in an abnormal manner so that many blood islands still remained and presented a bright red appearance. Six of the ten specimens had no circulation whatever and the entire arrangement of blood islands over the yolk was in exactly the same condition as on the day previous, except that the blood cells were now much redder in color.

During the course of the experiments similar tests and observations have been repeated four times. In each instance isolated groups of embryos showing yolk-sac blood island were selected and examined in order to ascertain on the following day the fate of these islands. In every case my experience was identi-

cally similar to that just described. The islands on the yolk-sac are often very far distant from the embryonic body as is readily seen by reference to figures 21 to 24. There is no doubt that wandering cells migrate out on to the yolk surface at a very early period and here give rise to blood islands comparable to those formed in other meroblastic embryos. It must be recognized, as I shall bring out in a consideration of the microscopic study of these embryos, that the yolk-sac of the fish is not entirely comparable to that of all other vertebrate types, yet there are many observations on the early living embryos which have convinced me that mesenchymal cells do wander from the embryo to various parts of the yolk-sac. These cells occupy a position between the ectoderm and the periblast (periblastic endoderm?) just as the peripheral mesoderm would in other yolk-sacs. Some of the wandering cells are future pigment cells of either the red or black variety to be mentioned later, others future endothelial cells, but many at least are to give rise to future blood cells.

Therefore, in embryos at about the beginning of the circulation one finds two distinct blood regions: The major region and most evident is the intermediate cell mass of former investigators, and the second position in which the blood cells are seen is the yolk-sac blood islands. The earliest yolk-sac blood islands are very easily overlooked. The writer had examined these embryos in great numbers and studied them for sometimes before finally discovering the existence of the early islands. With a high power single objective binocular, however, after their location is known the observer is readily able to see the nuclei of these cells in the posterior region of the yolk-sac, and they may then be followed from time to time. After these observations there is finally no doubt that blood islands do form on the yolk-sac of the Teleost embryo but these islands are probably to be regarded as disconnected portions of the intermediate cell mass or blood anlage.

It is freely admitted that this yolk-sac island blood formation may not take place in all Teleosts. The early wandering cells that here give rise to blood islands may in reality be compared

to the growing out onto the yolk of masses of cells from the intermediate cell mass as described by Swaen and Brachet ('99, '01). These cells in the species here studied may wander earlier and more freely than in the trout.

At any rate, as shall be brought out later, the ventral mesoderm of the yolk-sac in other vertebrates and the intermediate cell mass of the Teleosts are very closely related homologous portions of the mesoderm, if not one and the same thing.

4. The five-day embryos

The conditions which the embryos have attained five days after fertilization are illustrated in figures 1 to 4. In figure 1 a normal individual of this age is shown. The heart is slightly twisted but still more or less tubular. The vascular network on the yolk-sac is well established. The pigment cells are numerous but not yet fully developed and have not assumed an alignment along the blood vessels or taken on the usual embryonic pattern. The heart, of course, is pulsating vigorously and the blood current is easily seen both within the embryo and on the yolk-sac.

The three other figures in this group show different conditions of arrested development in individuals without a blood circulation. In figure 2 the pericardium is seen to be hugely distended so that the head is pushed or raised away from the yolk surface and the heart is stretched out into a long narrow tube extending from the ventral surface of the head to the sheer anterior surface of the yolk. This heart pulsates feebly and can be seen to contain a small amount of fluid which is churned up and down by the pulsations. None of this fluid, however, is ever pumped away from the heart. The pigment is much less plentiful than in the normal embryo of the same age, and the individual chromatophores are smaller in size than those of the normal embryo and have not sent out processes of any great length. No blood vessels at all are seen within the yolk-sac but very small scarcely noticeable blood islands are present on the posterior yolk region though not indicated in the sketch.

Figure 3 illustrates a more or less similar condition seen from a dorsal view. A small portion of the heart slightly projects beyond the anterior end of the head. If this egg were placed in lateral view, as was the case in figure 2, then the heart would be seen to show a similar condition, since it also was stretched out into a long narrow tube.

Figure 4 represents a very defective embryo. Specimens similar to this occur in great numbers in the stronger alcohol solutions. The bodies are very short since the descent of the germ ring over the yolk sphere is slow and at times incomplete, and the tail end of the embryo is often bifid or split giving a condition of cauda-bifida. At the anterior end, the upper right side of the figure, is shown the distended pericardial vesicle, and at the posterior end another large distended vesicle is in most cases the Kupffer's vesicle, but in some instances this is possibly distended spaces in the yolk mass just below the Kupffer's vesicle. These two sacs or spaces at the opposite ends of the body seem to be the places in which the non-circulating plasma most often accumulates to a great degree, in fact the accumulation of plasma is the actual cause of the exagerated condition of the spaces. In the individual illustrated by figure 4 the chromatophores are extremely small, but have arranged themselves to some extend so that they are very abundantly accumulated around the periphery of the Kupffer's vesicle while others have collected in the region of the pericardium. In the lateral yolk regions there are scarcely any pigmented cells present. All of these chromatophores, however, are small and contracted with very few processes of any extent.

In Fundulus embryos there are readily seen two distinct types of chromatophores. The one is a large dense perfectly black body with short broad processes. While the second is of a reddish color at first small and without processes, but later sending out very long graceful radiations which grow at the expense of the central mass until finally the whole chromatophore assume a moss-like branched structure, figure 6 shows both types well expanded.

Loeb ('93) at one time pointed out that these chromatophores migrate to the blood vessel walls and thus map the circulation

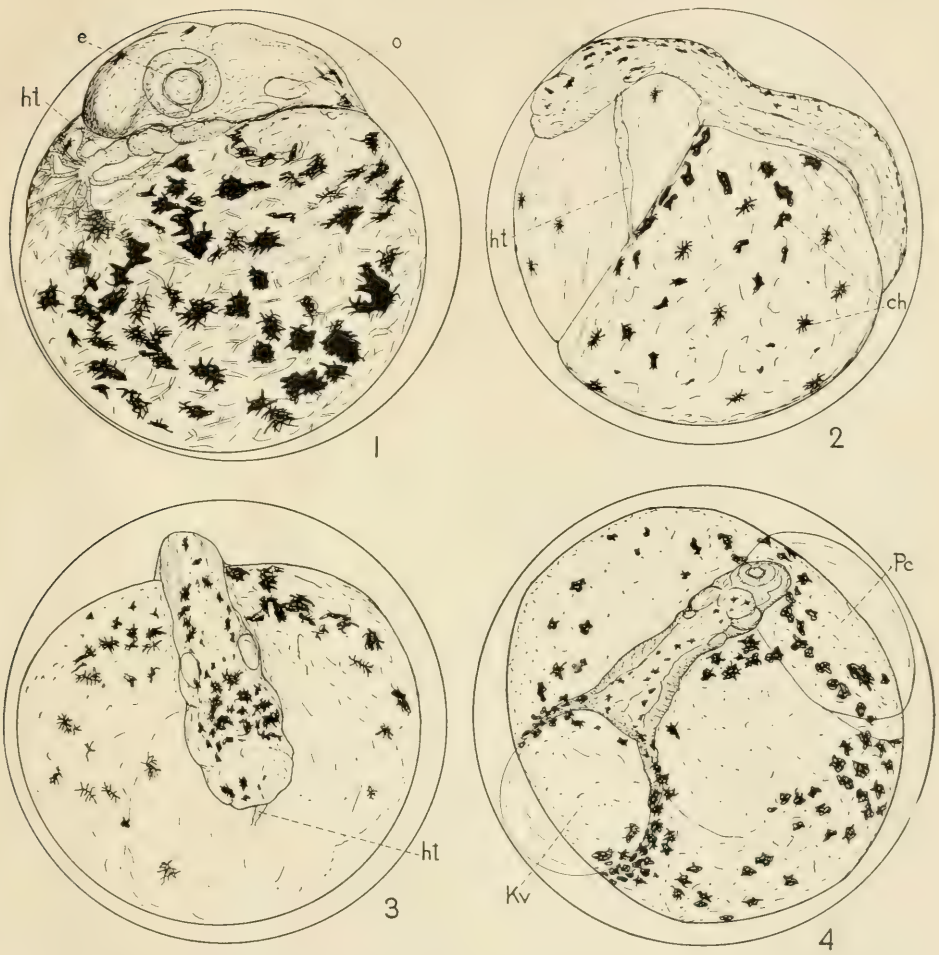

Fig. 1 The head end of a normal Fundulus heteroclitus embryo from life, five days old; *ht*, the heart, showing its S-shape; *e*, eye; *o*, ear.

Fig. 2 From a living embryo of the same age, developed in a weak solution of alcohol; the blood does not circulate and the body is small; *ht*, the heart stretched into a straight tube surrounded by a much dilated pericardium; *ch*, small and unexpanded chromatophores.

Fig. 3 The head end of a five-day embryo without a circulation, from a weak alcohol solution; *ht*, the heart slightly projecting from beneath the head.

Fig. 4 A five-day embryo from a stronger alcohol solution; the eye is cyclopean, the posterior end of the body split, cauda-bifida, and no blood circulation. Pigment cells are collecting about the sinuses; *Pc*, distended pericardium; *Kv*, Kupffer's vesicle, also hugely distended.

on the yolk-sac of a normal embryo. While in embryos treated with KCl in which there was no circulation, the pigment cells failed to asume any definite pattern. They remained more or less indefinitely scattered over the surface of the yolk and the body of the embryo in no way tending to align themselves along the vessel walls. From this fact, Loeb concluded that it was probably due to some chemotactic reaction that the pigment cells lined up along the blood vessels when the blood began to circulate and the attracting substance was possibly the oxygen contained within the blood corpuscles. Observing the various individuals without a circulation which we shall here consider, it will be seen that the pigment cells have a strong tendency to migrate to any cavity filled with plasma or fluid, and it is not probable that this plasma or fluid contains any more oxygen than is present in the other portions of the yolk-sac or body. It would, therefore, seem more likely that some constituent of the plasma itself and not the oxygen contained within the blood cells was the stimulating principle which caused the migration of the pigment cells to the vessel walls.

5. The eight- and ten-day embryos

The next group of figures illustrates the advanced condition which the embryo has reached by the eighth day. Figure 7 represents a normal Fundulus embryo of this age. The body

Fig. 5 An embryo eight days old, without a circulation; the heart, *ht*, poorly developed, beats feebly twenty-eight times per minute, about one-quarter the usual rate; *Pc*, pericardium greatly distended with fluid; from a "1.5 cc. alcohol solution."

Fig. 6 Eight-day embryo without a circulation, *ht*; the heart dilated with plasma pulsates ninety-five times per minute; *RCh*, the red chromatophores beautifully expanded, but no vessels present on the yolk. *Coe*, the lateral coelomic cavity dilated with fluid; *icm*, the intermediate cell mass now a great string of red blood corpuscles.

Fig. 7 A normal eight-day embryo, the heart, *ht*; pulsating rapidly and the network of yolk vessels mapped out by the chromatophores.

Figs. 8 and 9 Two eight-day embryos without blood circulation; chromatophores unexpanded but collected on the heart, *ht;* the normal heart has no pigment cells on it; *Pc*, the dilated pericardium; *icm*, median mass of erythroblasts; *CV*, cardinal vein containing erythroblasts.

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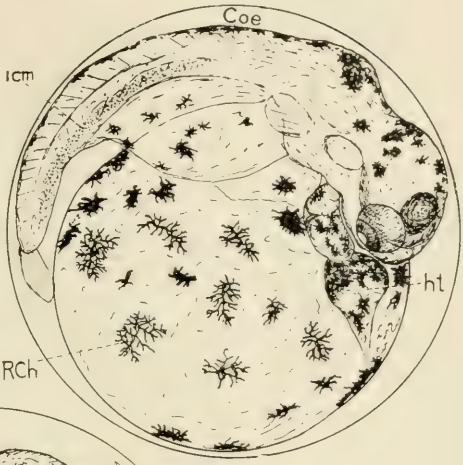

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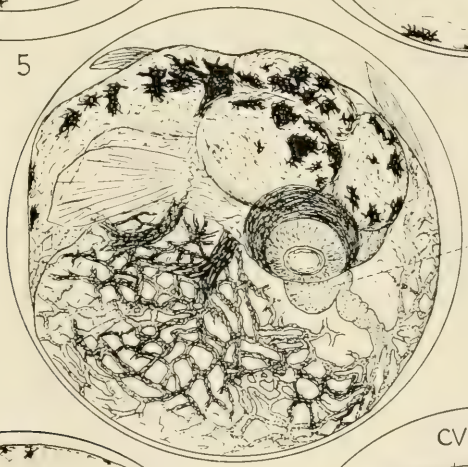

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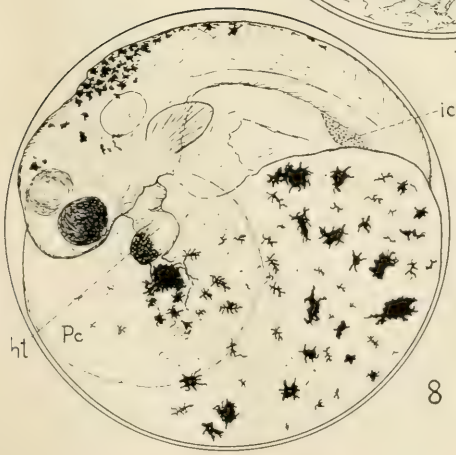

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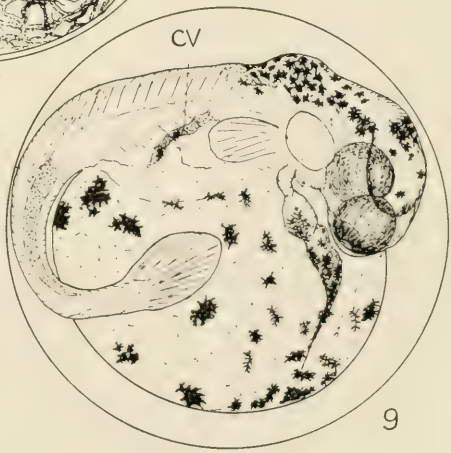

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is seen to be well developed, the fins are already capable of movement, and the brain and spinal cord are well shown and covered with the black type of chromatophores. The heart is seen to be more twisted than in the younger embryos and now occupies a position further under the head of the specimen. The network of vessels on the yolk-sac is beautifully mapped out by the arrangement of the pigment cells, largely the red type of chromatophore. It is to be especially noticed that pigment cells are never present on the heart of the normal embryo.

The other four figures of this group show individuals in which there was no circulation of the blood, although the hearts pulsated in a more or less feeble manner. In figure 5 the greatly distended pericardium is again shown, the heart is stretched from the head to the anterior surface of the yolk, and the lower part of the heart is completely sheathed with pigment. All of the pigment cells, however, are small and unexpanded.

In figure 6, the heart is very greatly distended and filled with plasma, yet it is apparently closed at one end since the plasma is churned up and down and never pumped out of the heart. In this case, there were several cells or particles suspended in the plasma contained within the heart, and these particles could be watched for long periods of time constantly moving up and down but never going out of their confined position. The pigment cells in this individual are greatly expanded, the red type chromatophores showing beautiful mossy processes. The lateral body cavities, *Coe*, or the coelomic spaces formed between the layers of the lateral plates of the mesoderm are greatly distended with plasma. A condition particularly noticeable in many such individuals. Red blood corpuscles are distinctly seen throughout the entire extent of the intermediate cell mass as indicated in the figure by the stippling in the posterior region of the body and the tail. The heart of this specimen is also richly covered with pigment and thus presents a striking contrast to the normal heart in figure 7.

In figure 8 much the same condition is presented except that here again the pigment cells are still contracted. The pericardium, however, is distended and the heart is covered with pig-

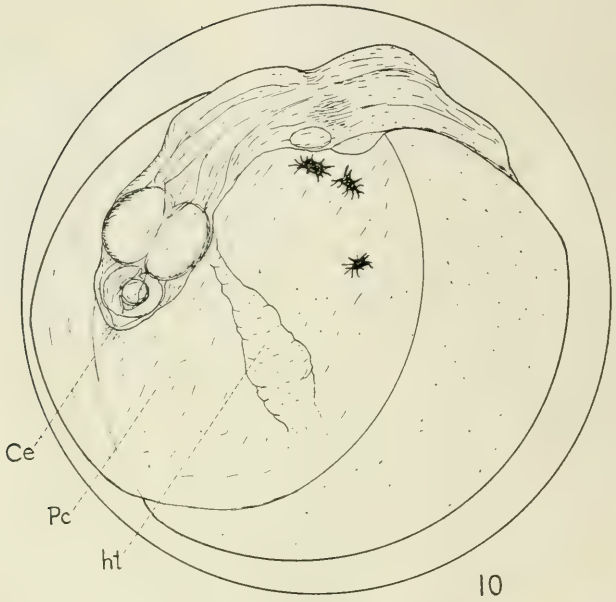
ment. The anterior end of the intermediate cell mass showing erythroblasts is just seen where the body of the embryo turns over the yolk sphere.

Figure 9 illustrates a lateral view of a small embryo. In all of these embryos in which the blood fails to circulate the fins are much smaller and less well developed than in the control, the entire body of the embryo is smaller and the whole appearance is that of a general developmental arrest, the rate of development being behind the normal. Yet such individuals have rather perfectly formed bodies, are capable of movement and seem in general to be very well developed, their only defect, so far as can be determined in many cases, is the absence of the circulation of the blood.

In figure 9 the heart is again sheathed with pigment cells, the blood cells in the intermediate cell mass are very distinctly present in the posterior body region, and in this individual a lateral vein in the position of the posterior cardinal also contains blood corpuscles. This appearance is seen in a number of individuals and may merely result from the fact that in these the intermediate cell mass is bilateral or split rather than entirely median in position. Such an explanation will seem probable, I think, after a consideration of the embryos in section.

Figures 12, 13 and 14 show three individuals of ten days old. These happen to be more or less abnormal. Figure 12 has very small eyes but the general body structure and shape are fairly normal. The pericardium is dilated, and the heart is small and pulsating feebly with a little pigment towards its aortic end. There is a great accumulation of pigment cells around the posterior region of the yolk sphere and near the distended Kupffer's vesicle in this embryo. Here again the cardinal veins are seen to be loaded with blood and only in the posterior body region do the two lateral masses come to unite into a median cell mass. Figure 13 shows much the same conditions, the heart is a mere filament indicated by the chromatophore along it.

Figure 14 gives a dorsal view of an embryo of ten days. The pigment spots are very few in number and the embryo has a pale



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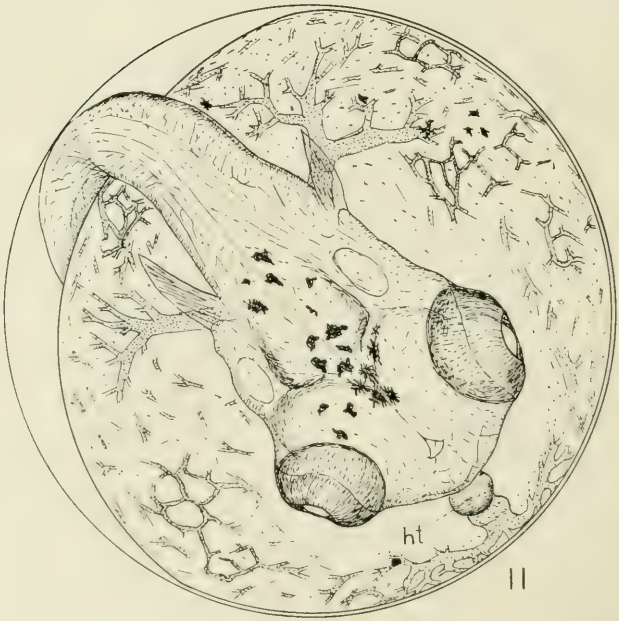

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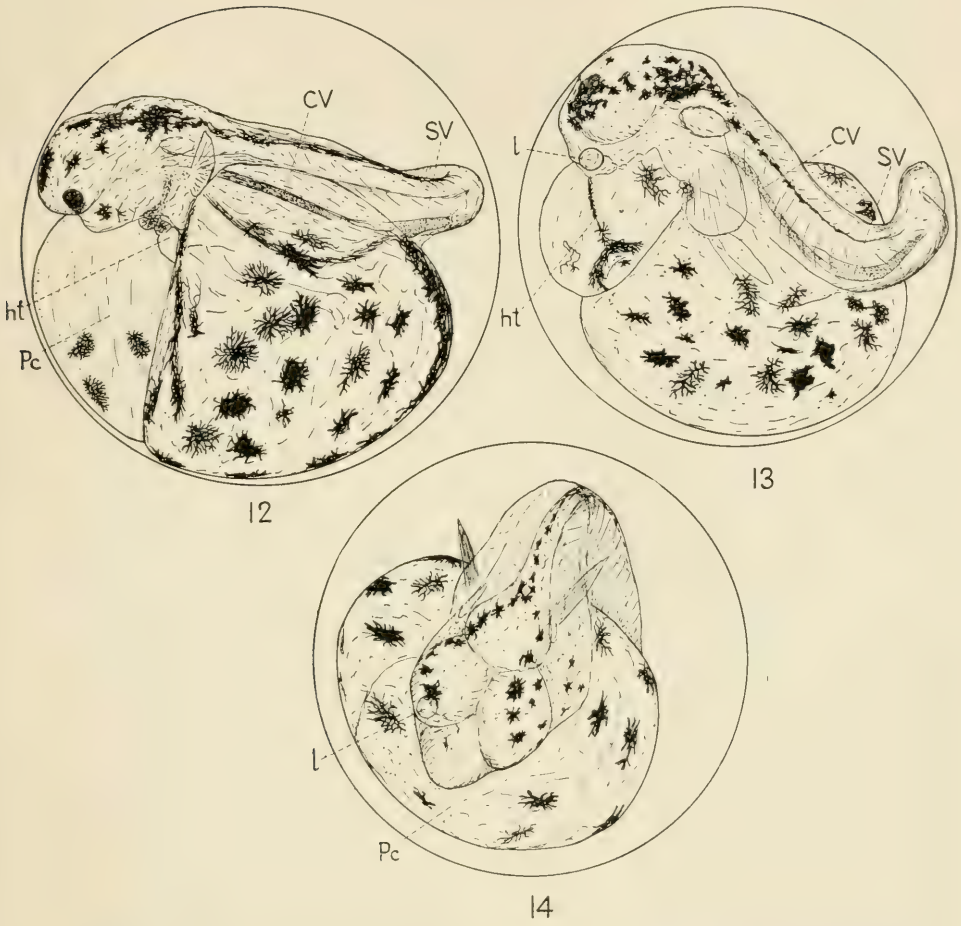


Fig. 10 An eight-day embryo of Fundulus majalis from 2 cc. alcohol solution, showing a condition similar to the heteroclitus embryos; *Ce*, cyclopean eye; *Pc*, distended pericardium; *ht*, straight heart and no circulation.

Fig. 11 A normal majalis embryo of eight days with S-shaped heart, *ht*, and yolk vessels forming a net. Same magnification as the smaller heteroclitus embryos.

Fig. 12 A ten-day heteroclitus embryo, no blood circulation, chromatophores expanded and accumulated on posterior yolk region; *ht*, heart; *Pc*, distended pericardium; *CV*, cardinal vein filled with erythroblasts; *SV*, stem vein also full of red blood corpuscles.

Fig. 13 Embryo ten days old; *ht*, the heart a mere string covered with pigment; *l*, an independent crystalline lens; other lettering as in figure 12.

Fig. 14 View of head of ten-day embryo, no circulation, distended pericarddium, *PC; l*, free lens.

appearance when compared with a normal individual, such as the one of eight days shown in figure 7.

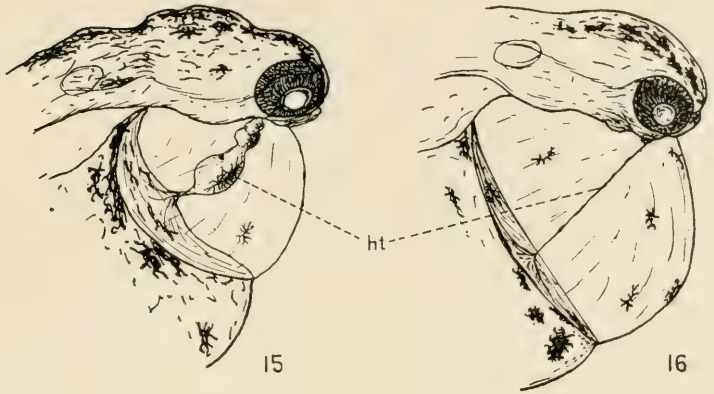
Figures 10 and 11 illustrates two specimens of Fundulus majalis drawn to the same scale as the previous figures. The egg of this species is considerably larger than that of heteroclitus, but its response to the experimental treatment is the same.

Figure 11 represents a normal embryo eight days old. It is seen not to be comparatively so far advanced at this period as is the heteroclitus, since its development is much slower and it requires from five to ten days longer to hatch. Very little pigment is present, yet the vessel net is well formed on the yolk-sac and the heart is distinctly seen to be more or less S-shaped.

Figure 10 is an embryo of the same age that had been subjected for forty-eight hours to a solution of 2 cc. of 95 per cent alcohol in 50 cc. of sea-water. Scarcely any pigment is present, the pericardium is typically distended and the heart is stretched into a long conical shape. No vessels are seen upon the yolk. The posterior end of the embryo is not shown in the figure, but in it could be seen early blood cells in the intermediate cell mass while a few small blood islands were present on the yolk-sac near the posterior end of the embryo.

After these eight- or ten-day stages very few changes of interest take place. The normal embryos hatch at from eleven to fourteen days as a rule, and become free swimming. The individuals without a circulation of the blood never succeed in breaking out of the egg membrane but may remain alive for twenty-five or thirty days in some cases, and almost all of them will live at least sixteen to twenty days. The red blood corpuscles are very distinctly noticeable in these older individuals and can be seen to remain permanently in their original places of origin. The intermediate cell mass may cease to be distinguishable, as was evident in the old specimens. This, however, is not due

Figs. 15 to 20 Living embryos sixteen days old, without blood circulation, showing variations in the pericardial distension, the position of the heads, and the perculiar heart conditions. These hearts, *ht*, all pulsate feebly and in several figures the slight lifting of the anterior yolk membrane is shown; this small membranous cone is rythmically raised with the pulsations.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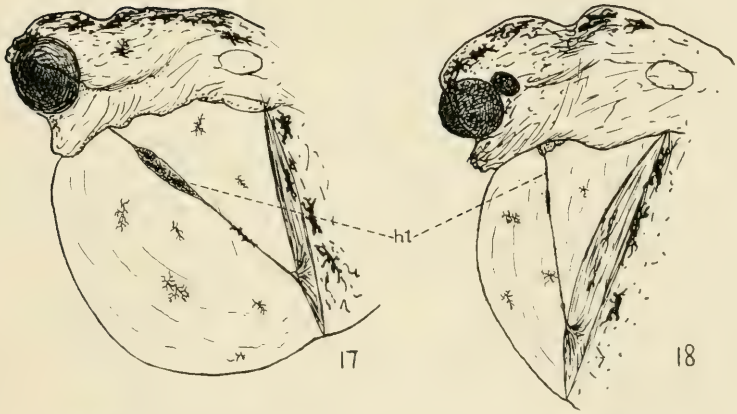
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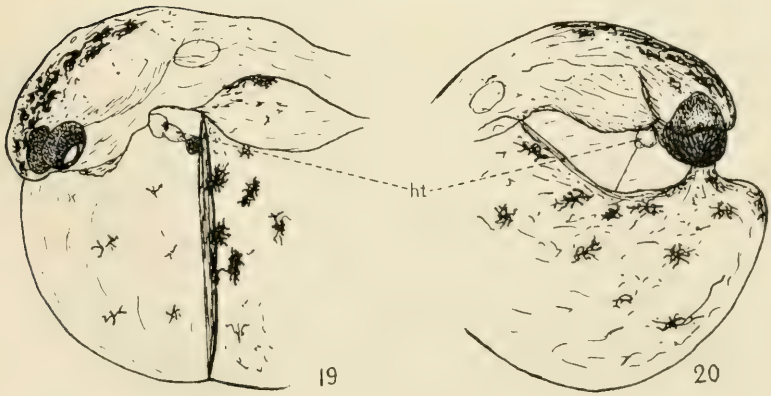
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20

to the blood cells having wandered away from the mass since they have largely degenerated *in situ* probably on account of lack of aeration. The blood islands on the yolk-sac maintain their red color for a much longer period of time, and they continue to present a pattern closely identical with that seen in the same individual during its earlier stages.

6. Condition of the heart in old embryos without a circulation

The conditions of the heart in some of these old embryos is well shown in the series, figures 15 to 20. These figures are from embryos of sixteen days old. The control specimens at this time would as a rule have hatched. In the sketches the peculiarly distended pericardium is strikingly shown. This great distention of the pericardium seems to have exerted pressure in such a way as to have straightened the anterior end of the embryo and lifted it well away from the yolk surface. The mechanical pull caused by the separation of the head from the yolk would seem to be largely responsible for the fact that the heart becomes stretched into a very much attenuated tube or string.

In the upper left hand figure 15, the heart is not so greatly stretched and the pericardium in this case is not distended so much as in the others. The upper right hand figure 16, and the two central figures, 17 and 18, show the pericardium distended to its utmost, and in these specimens the heart is pulled out into a mere string. Pigment cells seem invariably to wander along these string-like hearts, and they, therefore, stand out in the embryos as a black cord just as is indicated in the figures. The venous end of the heart which is connected with the yolk-sac is seen at each pulsation to lift slightly the yolk membrane in a cone-like projection from the surface of the yolk. As these hearts pulsate in their feeble fashion, one thus observes the yolk membrane as it is pulled up and down.

The two lower figures, 19 and 20, show other somewhat different conditions of the heart. The hearts are small and do not reach so far towards the ventral portion of the yolk.

There is an almost limitless variety of peculiarly abnormal hearts in these embryos and the six figures convey but a slight idea of the many very strange conditions which are presented.

7. Development of the yolk-sac blood islands in life

The blood islands in the living embryos, as was mentioned before, are quite difficult to see in the early stages. But a few hours before the heart begins to pulsate and the circulation bebecomes established they are very evident in the posterior ventral yolk regions. The arrangement of the blood islands display various patterns in different individuals in some being inconspicuous while in others an extensive network is present. These blood islands probably arise largely from wandering mesenchymal cells since the yolk-sac of the Fundulus embryo consists at first only of the yolk periblast with the ectoderm immediately above it. There is no true mesodermal layer to the yolk-sac and this mesenchymal blood formation on the yolk can, in all cases, be traced to the early wandering cells.

In figure 21 the posterior end of an embryo of ninety-six hours is shown. The Kupffer's vesicle, *Kv*, is dilated and pigment cells have accumulated around it. Immediately posterior to this are a number of blood islands indicated by stippling.

Figure 22 shows an embryo eight days old with the posterior ventral surface of the yolk well covered with blood cells. Erythrocytes are also seen in the intermediate cell mass. The blood in this embryo has never circulated and one can scarcely conceive that the blood islands on the extreme ventral surface of the yolk are due to the crowding out or pushing away of cells from the intermediate cell mass within the embryo. These cells are rather to be regarded as true yolk-sac blood islands which have arisen from early wandering mesenchymal cells probably in the beginning derived from same source as the intermediate cell mass.

Figures 23 and 24 show the caudal ends of two normal embryos of seventy-two hours. In these the heart has not begun to pulsate nor the blood to circulate, yet a distinct group of erythroblasts or early blood cells are seen already arranged on the yolk-sac in this posterior region.

In figure 24, it would look as though these cells had wandered out from and grouped themselves around the tail end of the embryo. At this period, seventy-two hours, the intermediate cell mass within the embryo is not visible in life.

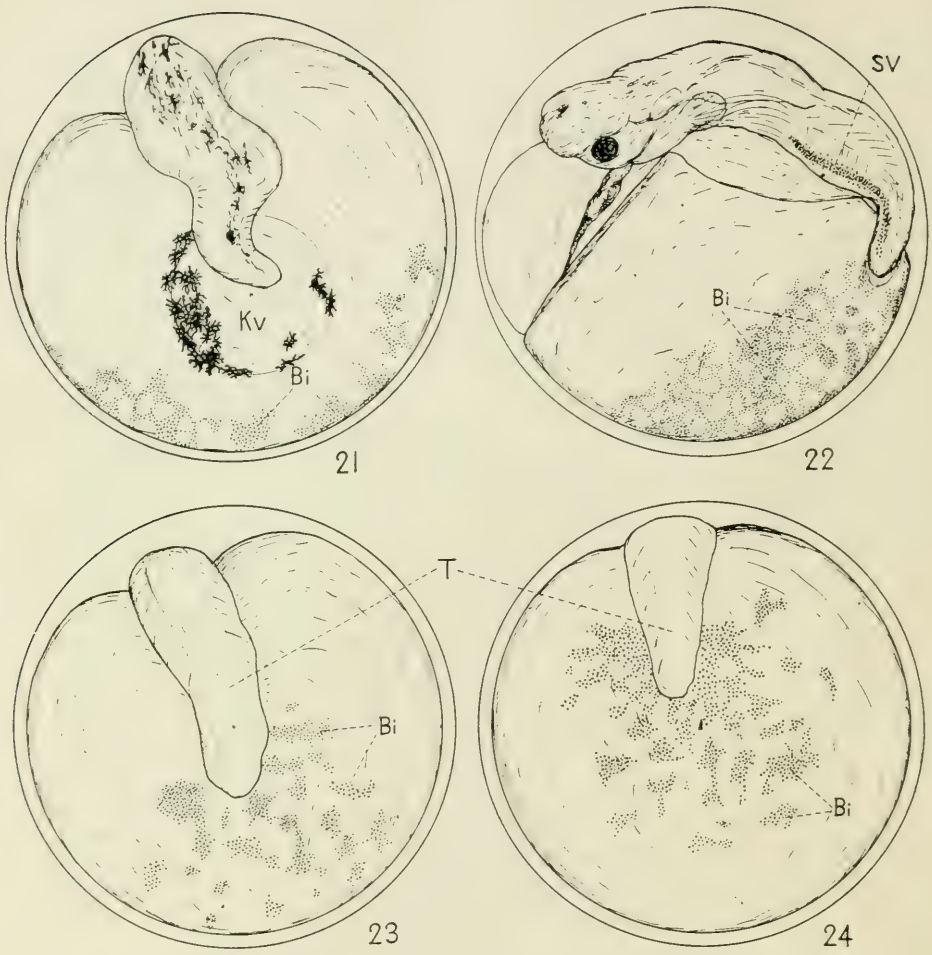


Fig. 21 Caudal end of an embryo of ninety-six hours, without a circulation; *Kv*, the distended Kupffer's vesicle with pigment cells collected around it. *Bi*, yolk-sac blood islands on the posterior yolk region.

Fig. 22 Lateral view of eight-day embryo, without a circulation, showing red blood corpuscles in the stem vein, *SV*, and also masses of blood islands, *Bi*, on the posterior ventral yolk regions.

Figs. 23 and 24 Posterior veiws of two seventy-two hour embryos, without blood circulation. Cells are seen wandering out from the tail, *T*, region into the position of the peripheral mesoderm in most meroblastic eggs; these cells collect into groups and form the blood islands, *Bi*.

It may then be concluded from a study of the living embryos with a circulation and others without a circulation, that in the normal ordinary individuals as well as in those having their blood flow prevented, the origin and formation of blood in the bony fish occurs as follows: The chief source of origin of the erythroblasts is that so fully described by previous investigators as the intermediate cell mass, la masse intermédiare. This mass, according to Felix ('97), Swaen and Brachet ('99, '01) and others, arises from the median portions of the two lateral mesodermal plates, primary seiten-platten. These bi-lateral masses migrate towards the middle line and there fuse to form the intermediate cell mass or blood string. In the living embryo this very important mass of blood cells is readily demonstrated. It is usually median in position, but in many cases, as illustrated above, it may be double or bi-lateral, at least in its anterior portion. This bilateral arrangement may possibly be the result of a failure of the blood forming portions of the two lateral plates to move to the middle line and fuse to form a Stammvene, in other words, a type of arrest.

The second seat of differentiation of red blood cells which is distinctly shown in living embryos is to be found on the yolk-sac in the posterior and ventral region where numerous typical blood islands form and develop. All recent investigators of the development of the blood in Teleosts have denied the development of blood on the yolk-sac. Most of their investigations have been on the eggs of the trout, and it may be that in this group there are no blood islands. But in Fundulus we seem to have a transitional condition in which the yolk-sac islands have not been firmly incorporated within the intermediate cell mass but still remain out or wander out upon the yolk. At any rate, we must conclude that there is a secondary seat of red blood formation in Fundulus embryos, and that in life it presents the typical appearance of yolk-sac blood islands.

From a study of the living embryos, it is apparently impossible to determine whether all cells of these blood islands are only erythroblasts or of mixed types. This is, however, readily ascertained by a careful study of sections.

THE ORIGIN AND HISTOGENESIS OF VASCULAR ENDOTHELIUM AND BLOOD CORPUSCLES AS DETERMINED BY STUDY OF MICROSCOPIC SECTIONS

1. The structure of the heart in embryos without a circulation

The hearts of the embryos in which there is no blood flow have been described in the living in the preceding consideration, but when they are studied in section an additional number of very instructive points are brought out.

In the first place, the heart wall is usually very thin and not well developed. This is particularly true, in the long string-like hearts that are present in those individuals in which the pericardium is so greatly distended. In the group of figures 25 to 28, one sees sections of these hearts taken through various regions.

Figures 27 and 28 show sections through a long narrow heart. In figure 28 the myocardium is seen to be practically one layer of cells, and within this the endothelial lining is distinctly formed. No noticeable structural difference beyond slight variations in shape can be determined between the nuclei of the mycocardium and those in the endocardium. The myocardial layer is a thick more or less structureless cell mass while the endothelium is well differentiated into a thin single cell layer lining. This condition is found in a non-circulating embryo of four days old. Tracing the series towards the conus end of the heart, we find the arrangement indicated in figure 27. The myocardium is here also a thick layer of cells enclosing a distinct endothelial tube.

Fig. 25 Section through the heart of a four-day embryo without a circulation; Experiment 11, 1912, Embryo 6. Heart wall poorly formed; large chromatophore, *Ch*, in wall; *ph.*, pharynx.

Fig. 26 Section of a similar heart; Experiment 11, 1912, Embryo 2. The guide outline gives the general relationships of the heart. *MC*, myocardium; *EC*, endocardium; *Br*, brain; *pb*, periblast nuclei, and, *pbs*, periblastic material filling the heart cavity, *c*; red staining cell.

Figs. 27 and 28 Through the aortic end and figure 28 through the tube-like body of a similar heart; Experiment 11, 1912, Embryo 7. *Br*, brain; *ph*, pharynx; *EC*, definitely formed endocardium, endothelium; *MC*, myocardium. The nuclei of the endocardium and myocardium are indistinguishable except for slight differences in shape.

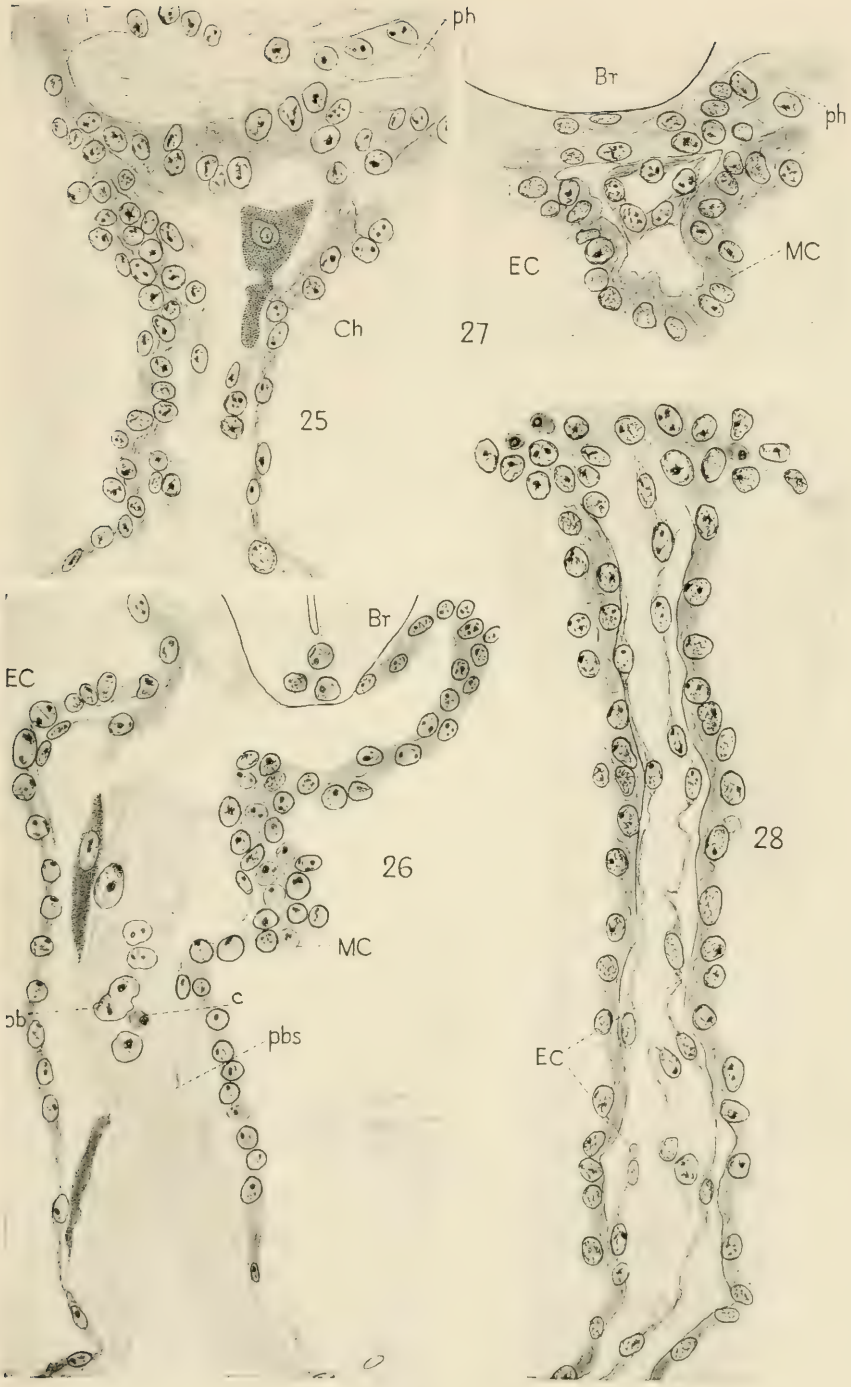

ph
Br
ph
EC
MC
27
Ch
25
Br
EC
26
MC
c
pbs
28
EC

Figure 25 is a section through another heart of the same age. In this a huge pigment cell is shown within the heart cavity. It is recalled that pigment cells were frequently seen to lie along these abnormal hearts while chromatophores were never present on the normal heart. The endothelium in figure 25 is more or less broken and the general condition of the heart is poorly developed.

In figure 26, a section is illustrated through a heart as it leads into the aortic arches. Here also large pigment cells are present. The endothelium is indicated in several places and within the cavity of this heart is a mass of periblastic material. It would look as though the periblast had been sucked from the surface of the yolk into the heart cavity. Several large periblast nuclei, *pb*, are indicated and are easily recognized on account of their amorphous shape and huge size.

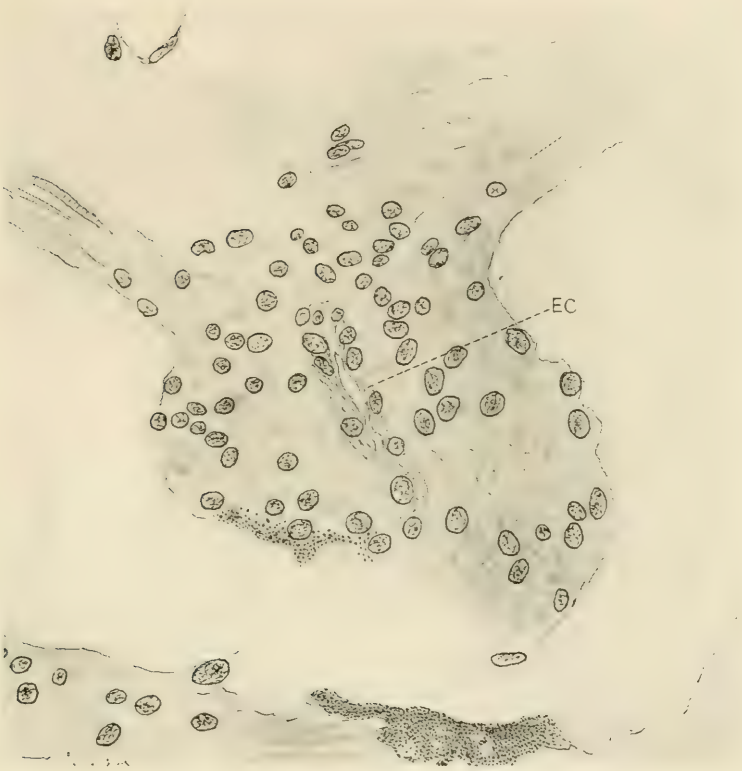
In figures 26 and 28 there are several cells, *c*, of a questionably degenerate type, the cytoplasm of which stains an extremely red color while the nucleus is small and pycnotic in appearance. These cells might in cases be looked upon as some type of wandering cell, but in most instances they are very degenerate in appearance.

It must be distinctly noticed that in none of the figures are erythroblasts shown. Throughout these heart regions at all stages the observer is impressed by the entire absence of any form of red blood cells in embryos that have absolutely had no circulation. One must constantly guard against the possibility of a slight circulation having existed for a short time and then having ceased. Another reason for blood movement may be the twisting or twitching reactions of the embryonic body. Conclusions regarding the permanent position of blood must

Figs. 29 and 30 Two sections through different parts of the heart in an embryo sixteen days old, without a blood circulation; Embryo 314.

Fig. 29 The aortic end of the heart, an almost solid mass with endocardial, EC, cells near the center.

Fig. 30 Through the string-like portion of the same heart; *pb*, periblastic nuclei and material completely fill the heart cavity; *EC*, endocardial cells; *Ch*, chromatophores surrounding the heart wall. The upper part of the section is cut slightly oblique.



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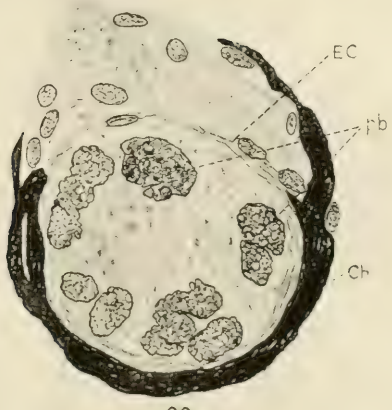

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be based only on embryos that have been carefully observed throughout their existence.

Figures 29 and 30 show sections through different parts of a solid heart string from an embryo of sixteen days old. In figure 29, the aortic end of the heart is shown to be almost a solid mass, and only near the center of the figure is a slight endothelial-like cavity or formation.

Figure 30 is a cross-section through the long string-like portion of this heart. It is seen to be completely solid, the central portion or core consisting of periblastic material containing large amorphous periblast nuclei, *pb*. Chromatophores have almost ensheathed the structure and present in the figure a dense black border. In one part of the section, however, a distinct endothelial-like formation is shown surrounding the periblastic core, and this heart again would seem to have sucked itself full of periblastic material from the surface of the yolk. As stated, this heart was from an embryo sixteen days old in which the blood had never circulated, and it is quite evident that at the time the embryo was fixed, it would have been impossible to have had a circulation of blood through such a solid heart. In this specimen, however, numerous blood islands on the yolk-sac and well formed blood cells in the intermediate cell mass were to be seen.

The endothelial lining of these hearts has certainly arisen *in loco*, and has emphatically not grown into the heart from the yolk-sac vessels since the heart is not connected with such vessels, and further, no typical vessels are present on the anterior portion of the yolk-sac. In all cases, the intra-embryonal vessels are much better developed than the vessels of the yolk-sac. A general survey of these embryos would quickly convince one that the vessels within the embryo are in no case derived from ingrowths. This fact is peculiarly emphasized in a study of bony fish embryos, and is so convincing that it led Sobotta ('02) to develop a theory of vascular outgrowth from intra-embryonic vessels in contrast to the older parablast notion of His ('75), but I must agree with Mollier ('06) in his view that both theories are equally untenable.

The hearts in these experimental embryos as a rule lead directly into a more or less well formed aorta which, in all cases shows an endothelial lining. The arches arising from this ventral aorta are very variable in the different embryos, yet in some cases are formed in an almost normal fashion. These arches also show a beautifully formed endothelial lining, but here again one is impressed by the absolute absence of erythroblasts in any stage of development within the neighborhood of the heart or aortic endothelia.

2. The "intermediate cell mass;" its origin, position and significance as an intra-embryonic blood anlage

On tracing the sections posteriorly one finds the intermediate cell mass to begin caudad of the pectoral fins and in the region of the anterior portion of the kidney duct. In studying a progressive series of very young stages forty-eight, sixty-six and seventy-two hours, the intermediate cell mass may readily be demonstrated to arise from the lateral mesodermic plates in the manner so clearly described by Swaen and Brachet ('01, '04). Felix ('97) previously pointed out that the primary lateral mesodermic plates extend away from the somite and later become divided into the following three parts. The median cells lying close to the somites separate away to form a continuous longitudinal string, the string from each side forming one lateral half of the future intermediate cell mass. The intermediate cells of the primary lateral plates just lateral to the above median group give rise to a second cord of cells which later forms the primary nephric duct. The remaining lateral layer of cells now constitutes the secondary lateral plates which split to form two lamellae.

The primary lateral plate mesoderm thus gives rise to the intermediate cell mass, the primary nephric ducts and the somatic and splanchnic mesodermic layers of the lateral body wall. Between these two lateral mesodermic layers arises the portion of the coelomic cavity which we have seen in the living embryos without a circulation of the blood to be greatly distended with fluid.

The later development of the intermediate cell mass is found to proceed in almost exactly the manner described by Swaen and Brachet ('01, '04). This mesenchymal mass of cells is at first of an indefinite type lying between the notochord above and the intestine below and being flanked on either side by the primary nephric ducts. The first notable differentiation of the intermediate mass in the normal embryo begins shortly previous to the establishment of a heart beat. In an experimental embryo of seventy-two hours old, that is one in which the heart was just about ready to begin beating, figures 31 and 32 show the condition of the intermediate cell mass in cross section.

In figure 31, which is the extreme anterior end of the mass and, therefore, less well differentiated than the more posterior regions, the cells are seen to possess large round nuclei differing but slightly from the nuclei of the surrounding cells and those of the epithelium of the Wolffin ducts. The mass of cells is completely unsurrounded by endothelium, and I agree entirely with Swaen and Brachet that the central cells go to form the red blood corpuscles while the cells about the periphery of this mass form the vascular endothelium.

Figure 32 illustrates a section through a more posterior region of the same embryo, the intermediate mass is seen to be much further differentiated. The cells are here typical early erythroblasts and many are observed to be in active mitosis. The cells in the mass are becoming dissociated so that they are no longer so densely packed as in the section through the anterior region. This section is posterior to the ends of the Wolffian ducts, as well as the closed intestine and beneath the cell mass is shown the periblast over the yolk.

On tracing the series still further caudad, we find the indefinite cell mass end Knospe described by Marcus ('05), figure 33. This is a ventral cellular mass into which leads the notochord, intermediate cell mass and end of the endoderm. In other words, this mass would seem to represent the end bud at the dorsal blastopore lip, as if it were the point from which differentiation had taken place or from which the layers had grown forward.

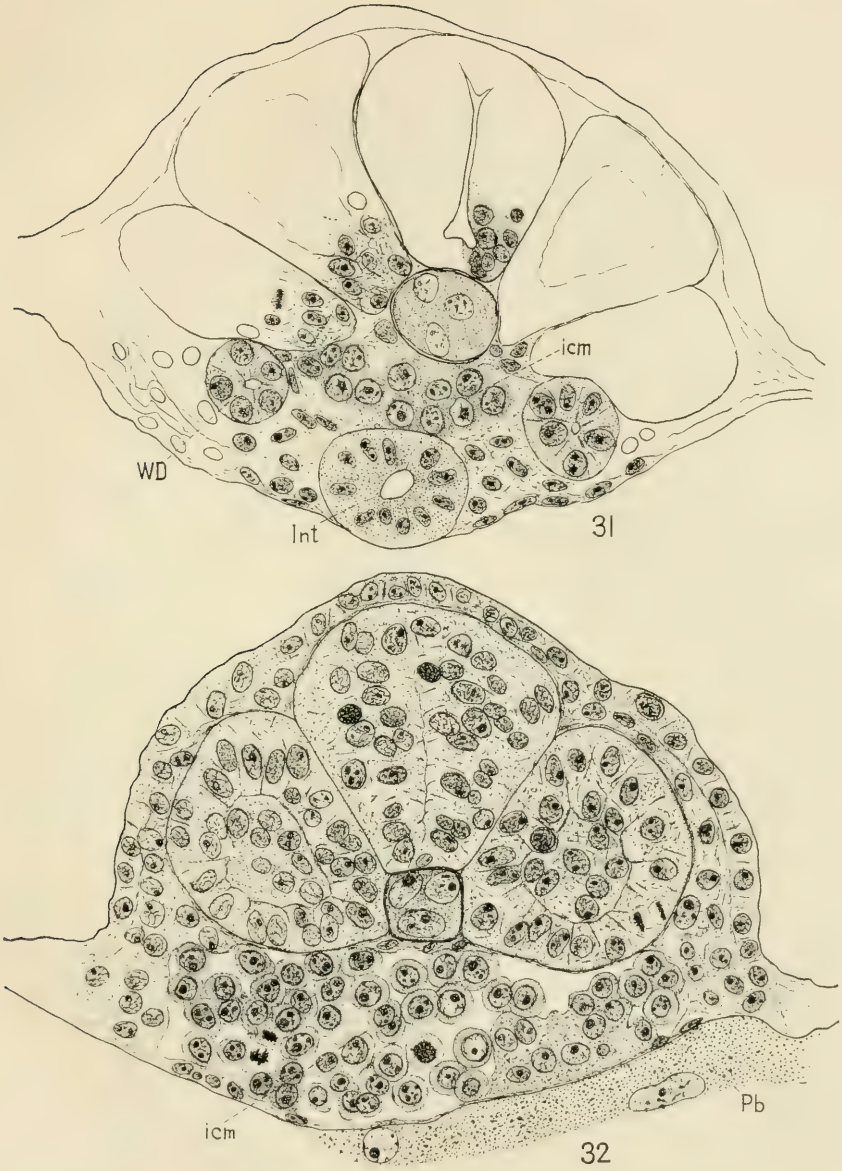


Fig. 31 Section through the trunk region of a seventy-two hour embryo without a circulation; Experiment A, 1913. The extreme anterior end of the intermediate cell mass, *icm*, is represented by deeper staining cells between the notocord and intestine, *Int;* the primary kidney ducts, *WD*, are lateral to the mass.

Fig. 32 Represents a more posterior section through the same embryo; in this region the intermediate cell mass, *icm*, is more extensive in cross-section and its cells are further differentiated than those in the more anterior region, *Pb*, periblastic material and large nuclei between the embryo and the yolk.

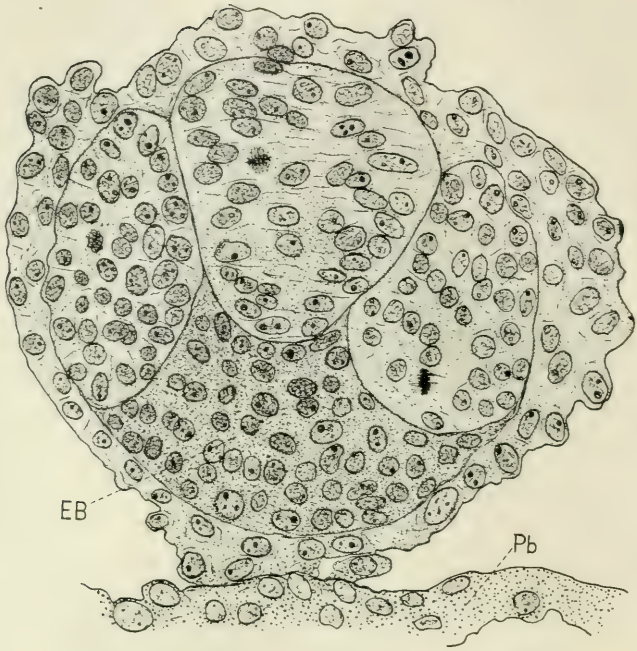


Fig. 33 A still more posterior section through the same seventy-two hour embryo as figures 31 and 32. This section is posterior to the place where the intermediate cell mass and gut endoderm fades out into the indifferent cell mass, *EB*, which may be considered to represent the end-bud mass; *Pb*, the yolk periblast.

The condition in the seventy-two hour embryo is, of course, quite early and the cells are not yet as a rule taken into the circulation. The appearance shown in these figures is exactly that of very slightly younger normal embryos in which a circulation would later be established. The figures, however, were made from sections of an embryo that had no heart beat at the time of its fixation, and, therefore, there is no chance that any of these cells could have become misplaced by having been circulated or carried about.

The most careful study with the highest power of the microscope has failed to reveal any type of cell in the intermediate cell mass other than the early erythroblast, and in later condi-

tions one finds here only red blood corpuscles. In other words, this chief stem blood anlage of the bony fish seems to be a specific red blood cell forming mass. This mass was first discovered by Oellacher in 1873 and has been shown by numerous investigators, Zeigler ('87), Winckebach ('86), Henneguy ('88), Sobotta ('94), Felix ('97), Swaen and Brachet ('99–'01) and others, to be peculiar to the Teleosts.

It is important to know that none of these investigators have yet recorded any type of blood cell arising from this mass other than the erythroblast. Of course, it may be argued that no special study was made of this particular point. Yet it is certainly true that if lymphocytes or leucocytes had been present to any extent, they should have been observed by many of these very capable and careful workers. It has recently been claimed by Maximow ('09), Dantschakoff ('07, '08) and others, that the popular opinion that leucocytes are very late in arising is erroneous since they actually arise just as early as the erythroblast. Then it seems all the more probable that if lymphocytes or leucocytes had been present in this intermediate cell mass such cells would have been discovered, since the mass has been carefully investigated right up to the moment at which it becomes swept into the circulating plasma.

Various investigators have differed as to the vascular products derived from the intermediate cell mass. Some have claimed that it forms only blood cells and no vascular endothelium, Sobotta ('02), while others have attributed the production of cardinal veins or venous endothelium as well as the blood cells to this mass, Felix ('97), and finally others, Swaen and Brachet ('01, '04) in particular, have considered this to be the source of both the aorta and cardinal veins as well as the red blood cells. I have spent considerable time in a study of this question and am inclined to believe that the endothelium of the cardinal veins and aorta arises from the mesenchymal cells surrounding the intermediate cell mass, which are different in nature from the cells actually constituting the mass. Yet it must be admitted that up to the present moment a complete demonstration of the origin of aortic endothelium from the cells about the periphery

of the intermediate cell mass has not been satisfactorily shown. This question will be more fully considered in another section.

In the embryos in which the red blood cells remain confined within the median region throughout life these cells develop in a normal manner and become completely differentiated into typical ichthyoid erythrocytes and exist as such for some time. Finally, however, for reasons at present impossible to state but likely associated in some way with an insufficient supply of oxygen, these erythrocytes begin to degenerate and in old embryos of sixteen to twenty days only a very few or in some cases none are left in the large intermediate vessel. Mesenchymal cells seem to wander into the mass of erythrocytes and may take part in their destruction.

Figure 41 is a section through the intermediate cell mass of a sixteen-day old embryo and presents this degenerate condition. The erythrocytes are all small and necrotic and many mesenchymal cells are scattered among them.

As we shall see below, the power of existence of the erythrocytes is very much stronger in the blood islands where aeration is no doubt considerably better than in the intermediate cell mass.

3. Blood islands of the yolk-sac, their origin and development

The question of origin of blood cells on the yolk-sac of the Teleostian embryo has been a much debated topic. Almost all of the earliest workers claimed that blood arose in the yolk-sac islands of the bony fish just as in other meroblastic eggs. The later workers, however, have denied this statement and hold that the bony fish forms an exception to the rule, and is the only type of meroblastic embryo in which blood cells do not occur in islands on the yolk-sac.

It has been frequently admitted by several recent workers that certain wandering mesenchymal cells do migrate to the yolk-sac from the embryo and there form isolated blood cells or small cell groups, but that this blood formation is insignificant in amount as compared with the great blood forming intermediate cell mass.

The yolk-sac of the bony fish is peculiar in this connection. In most meroblastic embryos there is a definite mesodermic layer or membrane between the ectoderm and entoderm of the yolk-sac, and it is in this mesodermal layer that the blood islands arise. When one examines the yolk-sac of the Teleost embryo, the mesodermic layer is found to be largely, if not entirely, absent. Thus, the ectoderm lies directly over the yolk periblast which may be considered to represent the primary entoderm. Between these two layers many long spindle-shaped mesenchymal cells are noticed on careful examination, but these cells in the specimens examined are never arranged in a definite continuous layer.

Goodall ('07) has recently stated that in the sheep embryo, the yolk-sac mesenchyme is not to be considered a continuous layer, but consists merely of diffusely scattered wandering mesenchymal cells. These mesenchymal cells in the sheep as in the fish finally collect into groups and such groups ultimately give rise to the blood islands. In the fish it would seem as though the entire ventral or yolk-sac mesoderm, the chief source of blood formation, had been in its phylogenetic development incorporated or drawn into the body of the embryo as the intermediate cell mass, and only a few cells lag behind or later wander out to form the collections of mesenchymal cells upon the yolk. Ontogenetically there is no longer any indication of a mechanical drawing-in process but the wandering out of cells may be readily observed. It is also easily conceivable that this condition probably differs in different species of Teleosts. Therefore, some species may really form no blood cells in the yolk-sac, while again others might have an almost complete mesenchymal layer in the sac and in such a case would probably give a typical blood island arrangement. Whereas, an intermediate condition would be well represented in the species of Fundulus here studied in which there are numerous disconnected wandering cells later grouping themselves to form the blood islands on the yolk-sac.

The appearance of the wandering cells as they radiate out from the caudal end of the embryo on to the yolk-sac is strikingly similar to that shown by the cells wandering away from the cen-

tral tissue mass in a living tissue culture. The cells are elongated spindle-form and all are moving straight away from their seat of central origin. This phenomenon is well illustrated by the numerous figures of tissues growing in culture media and I shall give illustrations of it in a special study of this subject now in preparation.

In all of the non-pelagic bony fish eggs investigated up to now, the chief blood forming cells are without exception the intra-embryonic intermediate cell mass, and this mass is claimed to form both vessels and blood. While in the pelagic type of bony fish egg the mass is usually concerned in the formation of vascular endothelium, and the blood cells only arise after the embryo is hatched and free swimming.

This peculiar specialization in intra-embryonic blood formation which seems typical for the bony fish has caused the yolk-sac formation of blood to be almost completely neglected or overlooked by recent investigators. Yet in the species upon which I have experimented there is no doubt whatever that blood islands do arise on the yolk and their origin is from the wandering mesenchymal cells. The wandering cells may be connected in some manner with the intermediate cell mass, yet the presence of the islands cannot be explained in the way Swaen and Brachet ('01) have attempted to account for the yolk-sac blood. They assume that the islands are pushed out laterally as branches or portions of the intermediate cell mass. In many cases no direct continuation of cells is traceable between the yolk islands and the intermediate cell mass and even in extremely young embryos yolk islands may appear on the ventral yolk surface at a great distance away from the intermediate cell mass.

The group of four figures, 36 to 39, indicate the progressive patterns assumed in life by these yolk islands. In the very early stage, figure 36 shows separate collections of cells here indicated by stippling. These groups then become confluent as in figure 37, then more or less net-like in appearance with certain nodes or portions thicker and darker than the general net. In these nodes cell proliferation or blood formation is more active. Finally a typical vascular network arises which goes to make up the capillary yolk circulation of the embryo.

These appearances, as stated above, are not readily distinguishable in the very young embryos, yet with a little experience and a high power microscope any one may convince himself that the blood island formation proceeds to a very definite and considerable degree in these embryos.

Figure 34 represents a cross-section through the yolk-sac of an embryo of seventy-two hours old. The ectoderm of the yolk-sac now becomes two-layered, this continues to thicken as age advances until finally in old embryos the yolk-sac ectoderm is many cells thick and often folded and complex in arrangement sometimes showing villus-like processes. Beneath the ectoderm a group of early erythroblasts or blood cells is illustrated. These cells lie immediately upon the yolk mass here indicated by the heavy dark granules. The appearance of the cells in this blood island anlage are closely similar to those shown in cross sections of the intermediate cell mass in figures 31 and 32. The cell nuclei and general cellular arrangements of the two tissues are seen to correspond in appearance, and the manner of differentiation followed in both cases is identical.

In figure 35, a group of five early erythroblasts are shown which were present in a neighboring blood island. They had loosened themselves from the general island mass and appear very much, if not exactly, similar to the early erythroblast seen separating themselves from the compact mass, the intermediate cell mass (fig. 32). The nuclei in all cases are typically those of early red blood cells and the cytoplasm just begins to stain a very pale pink color characteristic of the halo seen around the young erythroblast. All of the cells shown in these yolk islands, both in the earliest condition of the island and in the late old yolk vessels of embryos without a circulation are invariably of the erythroblast or erythrocyte type. In no case has any type of lymphocyte or leucocyte been present in these yolk islands except as late wandering cells.

Not all of the wandering cells which are found on the yolk-sac go to form blood cells since many of them are future chromatophores or future endothelial vessel cells. The types, however, are distinguishable in rather early stages and do not seem

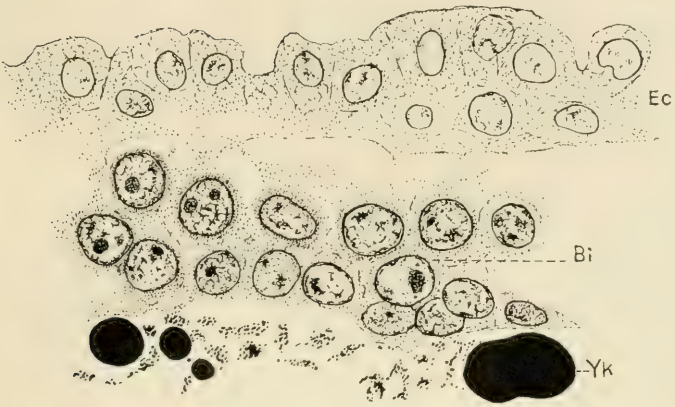
to be in any way related except that all are of mesenchymal origin. The chromatophores as before mentioned, often come to lie along the walls of the blood vessels.

The early yolk-sac is non-vascular, the blood masses being completely uncovered by endothelium. Later endothelial walls are formed around the blood cell masses and a vascular network is established in the yolk-sac of the normal embryo though poorly formed in the individuals without a circulation. All of these yolk vessels seem to arise by arrangement of wandering mesenchymal cells. Certain of these cells elongate and group themselves in such a way as to form vessel tubes. After the vessels are formed they may then be seen to send off buds and sprouts in the manner Clark ('09) has described in amphibians. The difference between the cells giving rise to the vascular endothelium and those forming the blood cells is not distinguishable in early stages. Yet after considerable study and careful observation, nothing has been observed that would indicate that these vascular endothelial cells possess the power to change into the blood cell type, nor is there any evidence to indicate that cells having once assumed even the earliest blood cell type are capable of metamorphosis to form endothelial cells. It is impossible to state emphatically that the vascular endothelium of the yolk-sac in all Teleosts arises in the same way as that described here for Fundulus embryos. But any one familiar with the very complex yolk circulation of the trout family, in the light of the above knowledge is scarcely justified in assuming that this network of vessels is completely derived from outgrowths from the aorta and cardinal veins within the embryo as Sobotta

Fig. 34 Section through the yolk-sac of an embryo seventy-two hours old, without a blood circulation. A group of cells forming a blood island are distinguished by a slight condensation of cytoplasm about their nuclei; Experiment A, 1913; *Ec*, the ectoderm several cells thick; *Bi*, the cells of the blood island; *Yk*, granular yolk.

Fig. 35 Young erythroblasts just isolating themselves in another island on same yolk as figure 34. Compare the early blood cells with those of figures 31 and 32, in the intermediate cell mass.

Figs. 36 to 39 Illustrate the progressive steps in the development of the network of yolk-sac blood islands.



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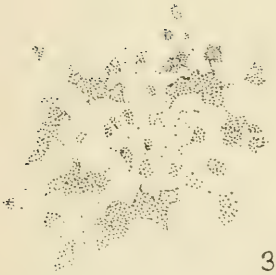

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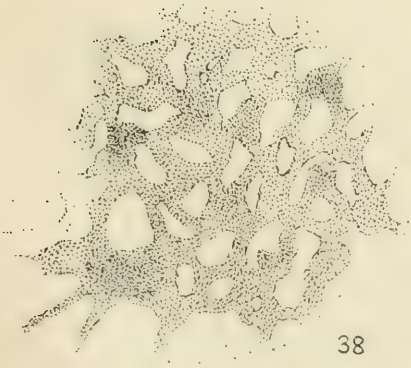

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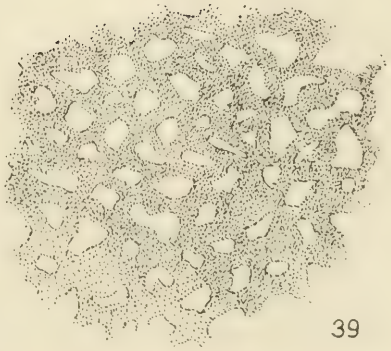

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('02) would have one believe. It is probably true that in the trout family also, wandering mesenchymal cells are of great importance in the formation of extra-embryonic vascular endothelium. There is a strong possibility, as admitted by Swaen and Brachet ('01) that some blood cells are also formed on the yolk-sac of the trout from wandering mesenchymal cells.

The blood cells in the yolk islands increase by mitotic division and soon become very prominent features in those embryos without a circulation, so that in old individuals of eight or ten days the entire posterior and ventral regions of the yolk are almost completely covered with red blood islands. The corpuscles in these blood islands persist in a more or less normal condition for a considerable length of time.

4. Fate of the blood corpuscles in embryos without a circulation

Figure 42 shows the corpuscles in a yolk-vessel of an embryo of sixteen days old in which the blood had never circulated. The vascular endothelium is well formed about the corpuscles and proliferation or multiplication of blood cells has completely ceased, the nuclei are very densely stained and somewhat pycnotic in appearance suggesting a more or less atrophic condition of these erythrocytes.

Figure 40 is a cross-section of a vessel from the yolk-sac of a normal embryo of seven days old. In this the vascular endothelium is also fully developed, large chromatophores have spread themselves along the vessel wall and the erythrocytes are in a vigorous physiological state. The nuclei are lightly staining alveolar structures quite different in appearance from those of the erythrocytes in the older embryo of sixteen days that has never had its blood to circulate. Yet the erythrocytes in the old non-circulating embryo are ladened with haemoglobin and certainly function to some degree.

Figure 41 in the same group is a section through the intermediate cell mass of a sixteen-day embryo without a blood circulation. This is the only intra-embryonic vessel which contains blood cells in this individual. The vascular endothelium

is completely disintegrated and has disappeared, and the very degenerate small erythrocytes are now intermixed with mesenchymal cells. In a slightly older embryo, all of these blood cells have disappeared within the tissue as if the invading mesenchymal cells had really assimilated or destroyed the old blood cells.

It is thus seen that in these non-circulating individuals, although red blood cells arise in a perfectly normal fashion and differentiate as completely as in the control embryos, yet they are not capable of maintaining their fully developed condition. Sooner or later they undergo degeneration and finally are completely absent from the body of the embryo.

It is noticed in all cases that very soon after the erythroblasts become completely surrounded by endothelium, they gradually lose their power of multiplication and then differentiate into typical erythrocytes. Before the vascular wall has completely enclosed the erythroblasts, all groups often show many cells in active mitosis, and as I shall bring out below those spaces in which blood cells multiply both in the embryo and in the adult are spaces not completely surrounded by vascular endothelium.

In examining figures 40 and 42, it may be of interest to note that the erythrocytes in figure 40 are the typical ichthyoid type of Minot ('11), while those in figure 42 are what Minot would describe or term, the sauroid type; that is, erythrocytes in which the nucleus has become slightly more degenerate or more densely staining than in the ichthyoid type and in which the cell body is smaller. This sauroid type of corpuscles Minot has designated as being characteristic of reptiles and amphibians, and the condition in these embryos without a circulation indicates the very artificial nature of the proposed classification of Minot. The cells are, of course, ichthyoid but are degenerate and, therefore, assume the 'sauroid type.'

It is difficult for one to believe that all of the functioning erythrocytes in the amphibians and reptiles really have a degenerate nucleus for the simple fact that mammals have blood corpuscles which have completely lost or discarded their nuclei. It must here be remembered that birds are as truly derived from

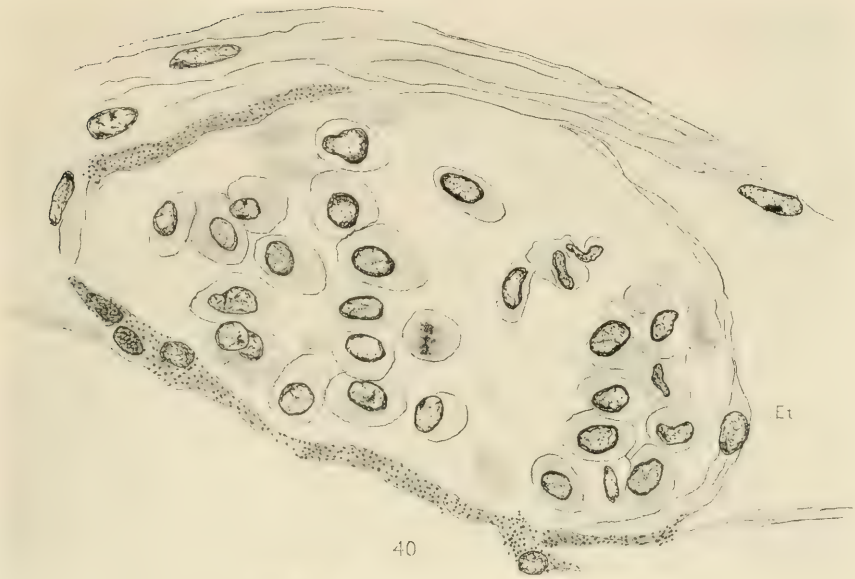
reptiles as are the mammals, in fact, the connection between the reptiles and birds is even closer. Yet the degenerating nuclei of the reptilian blood corpuscle is able to maintain itself in the corpuscles of the bird, although it is according to Minot, so far gone as to degenerate entirely in the corpuscles of the mammal. Such classifications are extremely misleading as they convey to the mind the impression that there is a continuous developmental or evolutionary chain of events illustrated in the blood cells of the different vertebrate groups and actually repeated in the development of the blood in the mammals. The "biogenetic law" is scarcely vigorous enough at present to be submitted to such a strain.

Finally in considering these yolk-sac blood-corpuscles, one must mention the possibility of origin from the yolk periblast or endoderm. It is often stated even in modern text-books and contributions that blood-cells may arise from endoderm and that the primary blood forming layer was actually the endoderm. It is very positively certain that none of the blood cells on the yolk islands of the fish arise from the periblast, but all are derived from wandering mesenchymal cells. The sharp distinction between endoderm and mesoderm is not a thing of any great or definite importance, since everyone recognizes the primary association and origin of mesoderm from the endoderm and the ectoderm. When the mesoderm is once formed, however, it contains within itself a blood forming anlage. It must be further remembered by speculators on the phylogeny of the vertebrate

Fig. 40 A highly magnified section through a yolk-sac vessel in a normal embryo of seven days; *Et*, the vascular endothelium with chromatophores along it. The large beautifully developed erythrocytes are seen in the lumen.

Fig. 41 An equally magnified section through the intermediate cell mass in an embryo without a circulation when sixteen days old; Embryo 413. This is the only intraembryonic blood present, the vascular endothelium, which probably at one time surrounded the erythrocytes, has broken down and mesenchymal cells, *Mcn*, are now intermixed with the small degenerate erythrocytes, *Ery*, which should be compared with the normal ones in figure 40.

Fig. 42 Shows erythrocytes in a yolk-sac vessel also in Embryo 413, at the same magnification as in the two preceding figures. These erythrocytes are in a better condition than the intra-embryonic ones, yet they are very degenerate as compared with those of figure 40; all, however, still contain haemoglobin.



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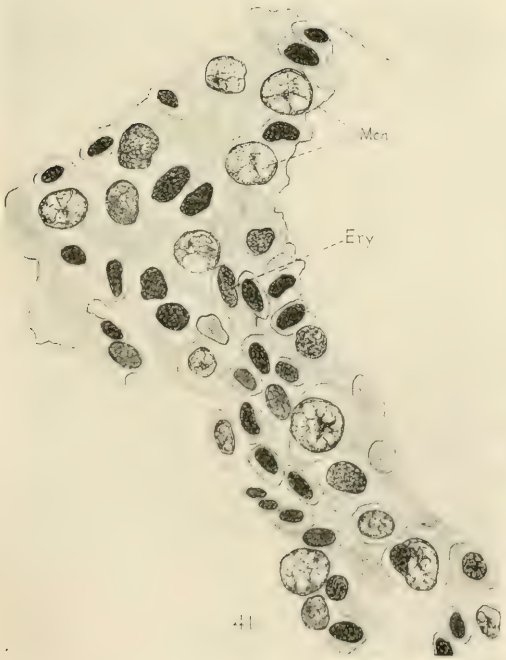

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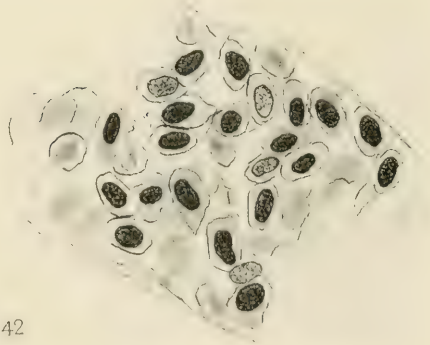

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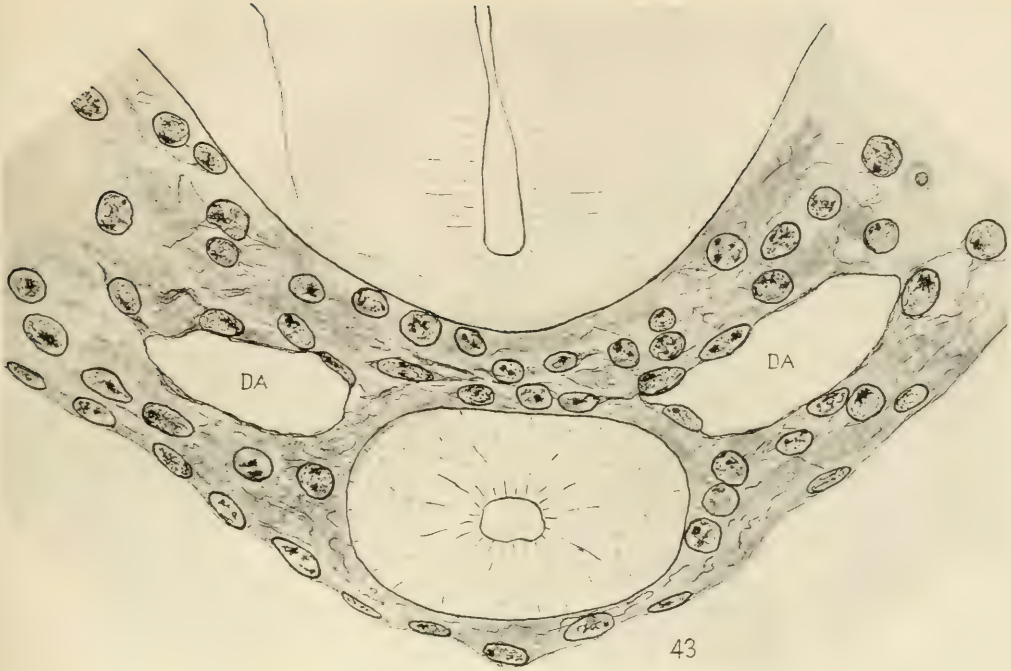
blood that the invertebrate animals, many of which possess highly functionating white blood cells, amoebocytes, as well as oxygen carrying corpuscles, are thought to derive these cells and the vascular endothelium from mesenchyme and not from endoderm.

5. Has vascular endothelium a haematopoetic power?

It has been mentioned in describing the origin of blood in various parts of these embryos that no observation could be interpreted to indicate that blood corpuscles ever arise from vascular endothelium. The endothelium of vessels containing blood never presents any cell in a transitional stage. These experiments, I think, furnish a crucial answer to persistent claims that vascular endothelium has the power to change into various types of blood corpuscles. If vascular endothelium had such a power, then one might expect that this power would show itself in cases where it was most needed, for example, in these embryos in which the blood has never circulated. The blood cells are confined entirely to the intermediate cell mass and to the blood islands on the yolk.

The heart and aorta and numerous vessels in the head and anterior portion of the body are lined with typical vascular endothelium, yet in no instance has it been found that one of these vessels contains a single red blood cell in any stage of development. From these experiments, one is warranted in making the bold assertion that the endothelial lining of the heart and aorta is perfectly incapable of giving rise to any type of blood cell. This fact has been mentioned in considering the endothelium of the heart. When we now refer to figure 43, a section through the anterior region of a four-day-old embryo without a circulation, two dorsal aortae are shown. These vessels are lined by typical embryonic endothelium but are completely empty so far as cellular elements are concerned. This is true of the dorsal aortae of all embryos from the earliest to the latest stages when the circulation of the blood has been prevented. Felix ('97) has also noted the fact that the aortae in early normal Teleost embryos are invariably free of blood cells.



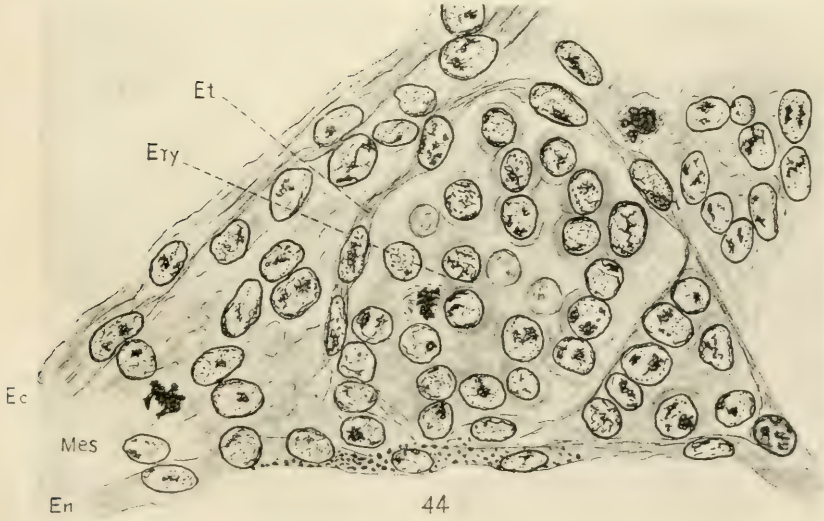


Fig. 43 Section through an anterior body region of a four-day embryo without a circulation; Experiment 11, 1912, Embryo 6. The dorsal aortae, *DA*, are seen lined by typical embryonic endothelium, yet they are throughout completely empty, never containing blood cells.

Fig. 44 A section through the cardial vein of a similar four-day embryo without a circulation; Experiment 11, 1912, Embryo 4. Again the embryonic endothelium, *Et*, is well formed but the lumen of the vessel is packed with erythroblasts; *Ec*, ectoderm; *Mes*, mesenchyme; *En*, entoderm.

Figure 44 accompanying figure 43 shows a striking contrast in the contents of the cardinal vein. This section is through a more posterior region of the same embryo. The vascular endothelium is here also well differentiated and the vessel is completely packed with early erythroblast some of which are still dividing. None of these erythroblasts, however, have been derived from the vascular endothelium and were actually present before the endothelium was differentiated.

The only source of intra-embryonic blood is from the blood anlage which is contained within the intermediate cell mass, as a rule. But in the splitting away of this mass from between the lateral plate and the somites, it is, of course, conceivable that some future blood forming cells might be left either in the somitic portion or the lateral plate portion. In such cases all those organs arising from regions which had been in contact with the intermediate cell mass either medially or laterally might be contaminated with blood forming cells. If the separation of the blood cell anlage takes place in a clean and complete manner in the individual embryo, then I believe the statement is true that all the intraembryonic blood will be contained in the intermediate cell mass or cardial veins which amount to the same thing.

6. The origin of lymphocytes and leucocytes or so-called white blood corpuscles

We may now turn to a consideration of the origin of lymphocytes and leucocytes or cells other than red blood corpuscles. Many authors have claimed from observations on various embryos that these cells are entirely distinct in their origin from the origin of the red blood corpuscles. Both types, however, arise from the same germ layer or mesenchyme. It has been repeatedly pointed out and seems to be thoroughly substantiated by fact that the lymphocytes and leucocytes in their first appearance are always interstitial in position and are only later contained within the vessels. Whereas, the erythroblasts are invariably formed or divided off into the vessels. In other words, the red

blood cell formation tends to be towards or into the vessels and the formation of white blood cells seems to be extra-vascular or interstitial.

It is recently claimed by Goodall ('07) that in the haematopoetic organs of the sheep embryo such as the liver, there are definite groups of proliferating cells forming the various types of white blood corpuscles, and these are distinctly isolated from other groups of proliferating erythroblasts. In the bone marrow this same state of affairs has been described, and in a number of diseased conditions of human marrow I have observed that certain nests or groups of cells were giving rise to leucocytes while other separate groups consisted of erythrocytes. This observational evidence might seem to indicate that white and red blood cells were arising from different parent cells. Yet in normal embryos it is very difficult to obtain material which will conclusively establish such a position, since both types of cells are swept around by the circulation and are intimately inter-mixed in all of the haematopoetic organs.

It would seem that in these experimental specimens in which the blood was prevented from circulating that there might possibly be some way to distinguish completely the source of origin of the white blood cells from the red blood corpuscles if these sources were really different. Should the two types of cells arise from the same common stem cell or parent cell, then the white and red blood cells should be invariably found in association in all embryos. If the two types of cells had different origins they might be found to occur in separate regions of the body and the various sources could thus be readily differentiated.

As frequently stated, in the early intermediate cell mass and among the cells immediately developing out of this mass no leucocytes or lymphocytes are found. The yolk-sac blood islands also consist entirely of cells of the erythroblast type. These observations are in accord with those of all other investigators studying the development of the blood in the bony fish. They have invariably described the intermediate cell mass as being the source of red blood corpuscles and no one has ever recorded either lymphocytes or leucocytes as arising from this mass.

The only cells within the embryo which resemble lymphocytes or leucocytes in their general structure and staining capacities have been found in the anterior portions of the body and in the head region of the young embryos. In very young embryos of seventy-two hours, numerous isolated cells and occasionally small groups of cells are found within the mesenchyme which present a peculiar appearance. The nuclei are more or less dense, the cytoplasm very small in amount in many and in others very extensive, and staining with a color quite different from that of other cells in the embryo.

Figure 45 shows a section through the head just behind the optic stalk of an embryo of seventy-two hours. In this section there is seen a nest of the above-mentioned cells, several are polynuclear and present various peculiar appearances. The mesenchyme within this region is in active mitosis.

Figure 46 is also taken from the anterior end of an embryo and shows two large mesenchymal nuclei with numerous small leucocyte-like cells within the mesenchyme. Numerous pigment granules are also present in these mesenchyme cells. Some of the cells present nuclei of the polymorphonuclear type.

Figure 47 shows an enlarged binuclear cell and indicates the fine granular nature of the cytoplasm. Such cells resemble very closely the embryonic white blood cells.

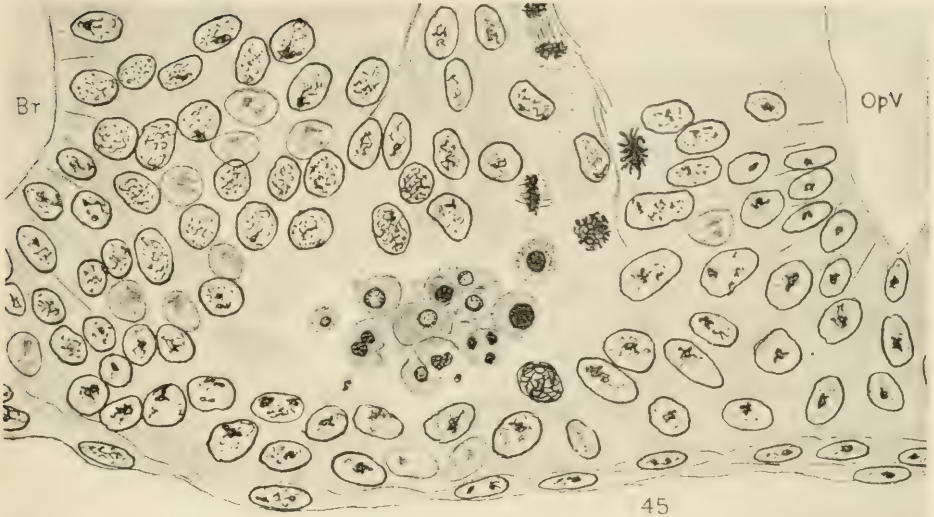
Figure 48 represents a section through the anterior end of an embryo, and shows an endothelial artery which is entirely empty of blood cells. Within the mesenchyme, near the vessel, are two of the leucocyte-like cells. Other cells in these embryos resemble very closely ordinary lymphocytes and these

Fig. 45 A section immediately posterior to the optic stalk in an embryo without a circulation when seventy-two hours old; Experiment A, 1913. A nest of peculiar finely granular cells lies in the mesenchyme which contains many dividing cells; *Br*, brain; *Opv*, optic vesicle.

Fig. 46 Cells from a four-day embryo; Experiment 11, 1912; *Mcn*, mesenchyme nucleus; small leucocyte-like cells are grouped in the neighborhood of chromatophores.

Fig. 47 A binuclear leucocyte from a sixteen-day-old embryo.

Fig. 48 Section through one of the dorsal aortae in a four-day embryo; Experiment 11, Embryo 6; embryonic leucocytes in the mesenchyme.



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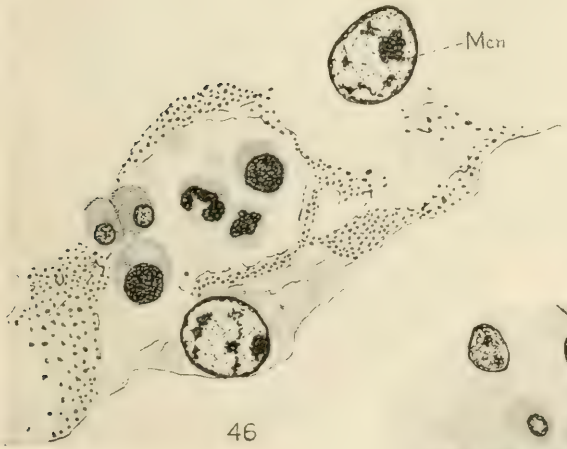

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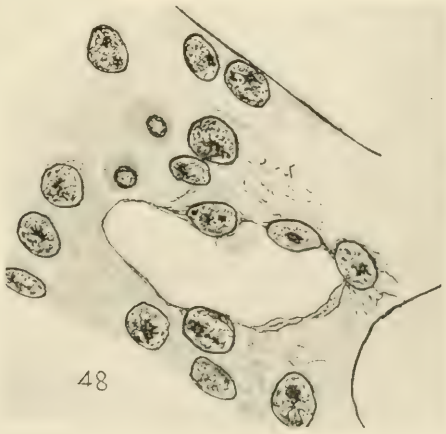

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also are within the tissue spaces and not in the vessels. None of these cells are found in associations with the early red blood cells.

I have examined a number of smears of heart blood, spleen pulp, bone marrow and peritoneal fluid from adult Funduli and have found within these specimens numerous coarse granular leucocytes, lymphocytes and various types of wandering cells. The embryonic cells above described all show a more or less degenerate appearance, but if they can be classed as any type of white blood cell their origin is definitely removed and entirely distinct from that of the red blood cell. In appearance they are as closely similar to embryonic leucocytes as are the cells designated by other investigators to be of that nature. It seems to me that the only possible method of differentiating between the origins of white corpuscles and red blood cells is to prevent their association in the circulating body fluids. These experiments along with numerous observations do show that the red blood cells arise in a region distinctly separated from those localities in which the white blood corpuscles are formed.

When one examines a specimen such as those in which Maximow, Dantschakoff and others have described the origin of white and red cells from a common stem cell, it is impossible to be absolutely certain that the two types of cells do arise from the same individual stem cell. The stem mother cell is shown within the mesenchyme, near this on the one side are various early lymphocytes or leucocyte-like cells, and on the other side are the various stages in erythroblast development. Each type of cell graduates directly back to the stem mother cell or to a mesenchymal cell. This much may be freely admitted, but to say more is merely a matter of guess or interpretation. Since it is absolutely impossible on fixed material to make the definite statement that both of these two types of cells have arisen from the one individual stem mother cell. The observer must actually witness the stem mother cell divide into two cells, and then observe one of these two cells either by differentiation or continued division give rise to white corpuscles, and the other either by differentiation or continued division give rise to erythro-

blasts before the existence of a common mother cell is proven. This very necessary observation has never been made and all of the evidence in the present literature seems insufficient to warrant the conclusion that such a thing does actually take place, whereas there is considerable evidence to indicate that white and red blood cells probably arise from two different mesenchymal cells. Of course, these two parent mesenchymal cells may be, so far as our powers of observation go, indistinguishable. Yet this would not indicate that they were not different in their potentialities. One of the two mesenchymal cells might be capable of giving rise to the various types of white blood cells depending upon the conditions of differentiation and function, while the other apparently similar mesenchymal cell could on account of its internal difference give rise to erythroblasts. It is a little strange at least that the white blood cells arise far interstitially, while the erythroblasts have such a decided tendency to proliferate into the sinusoids or vascular spaces if they both arise from a common stem cell. The two environments in which they develop could scarcely account for the differences between red and white corpuscles, since in the body of an embryo in which the blood circulates there are several places where the two types of cells develop side by side, as Maximow and others have described. The reasons for the differences are the internal differences between the mesenchymal cells from which the two types of corpuscles arise.

The white blood cells and red blood cells, although both are derived from mesenchyme, arise from mesenchymal cells which have already differentiated sufficienctly far not to be interchangeable. This statement is probably true also of the vascular endothelium in its relationship to blood forming mesenchyme. The embryonic mesenchymal cell if taken in an early enough stage could no doubt give rise to other mesenchymal cells which would later form any of these different type cells. When, however, differentiation has proceeded to some degree, sufficiently far to form what is termed by embryologists an organ anlage, and yet not far enough to make it possible to distinguish between the appearance of various mesenchymal cells, they are then, nevertheless, different in their potentialities.

The mesenchymal cell with the power of forming vascular endothelium is probably very diffusely distributed throughout the embryonic body as well as the yolk-sac. Numerous investigators have supplied evidence indicating this fact. On the other hand, the mesenchymal cells which are to form the erythroblasts are in the bony fish very definitely localized. The latter cells are chiefly confined to the intermediate cell mass, but in addition other erythroblast forming cells wander out probably from the same source of origin, the primary intermediate cell mass, to become distributed on the yolk-sac.

Finally, the mesenchymal cells which are to give rise to lymphocytes and leucocytes of various types seem in Fundulus embryos to be more or less localized in the head and anterior region of the body and do not seem to be particularly associated with vessels. For this reason in early embryos the first apparent lymphocytes and leucocytes are found in the head and anterior body regions, and even in older individuals such cells are more abundant here than in other portions of the body. Yet these cells are doubtless of a roving or wandering type and may finally become scattered throughout the embryo's body. While the non-migrating red blood corpuscles rarely if ever leave their original sites of differentiation.

7. Environmental conditions necessary for blood cell multiplication and differentiation

The above facts and interpretations lead us to a consideration of the later conditions of cell multiplication and differentiation. Are the so-called haematopoetic organs of the embryo and even the adult actually haematopoetic, or are they merely favorable localized environments in which various types of blood cells may multiply or reproduce themselves throughout the life of the embryo or individual? There is little doubt, from the recent suggestive studies on the cultivation of tissues in artificial media out of the body of the organism, that certain environmental surroundings are conducive to cell proliferation and growth while other environments inhibit these processes and tend to favor differentiation and functional activity.

Many points mentioned in the previous pages indicate that blood cells change their mode of behavior as the conditions of the embryonic vessels and body are changed during development.

A careful consideration of various embryos as well as the different regions of the same embryo suggests that erythroblasts only multiply in spaces unlined by endothelium, whether these be on the yolk-sac, or within the embryonic liver, spleen or bone marrow. . The unlined spaces thus afford an environment in which for physical or chemical reasons the erythroblasts are able to multiply and reproduce themselves. When, however, such spaces or channels become converted into endothelial lined tubes then the erythroblasts tend to differentiate into erythrocytes and very soon cease to reproduce themselves any further in this location. In the intermediate cell mass of the fish embryo for instance, one notices in early stages many dividing erythroblasts. Just about the time that this mass becomes completely enclosed by vascular endothelium, this division process slows down and finally ceases although the confined erythrocytes may never be able to leave the vessel. Soon after being surrounded by vascular endothelium the erythrocytes assume a passive non-productive state and remain in this condition throughout their existence.

One of the earliest places of blood cell proliferation or haematopoesis is the sinuses on the yolk-sac. Almost as soon, however, as these sinuses become converted into the yolk vessels, blood cell production ceases in the yolk-sac and only blood circulation takes place.

The haematopoetic processes are then transferred to the embryonic liver and this in most vertebrate embryos is an important seat of blood cell multiplication. The multiplying erythroblasts are never enclosed by endothelium. In other words, they are not within the vessels but their final products are invariably budded or divided off into the sinusoids. Finally, the spaces in which blood cells multiply in the liver become obliterated or converted into endothelial lined vessels and spaces and very soon after this takes place the haematopoetic processes cease in this organ. The liver cells themselves or the interstitial tissues of

the liver are not blood forming cells, the only blood formed within the liver arises from existing blood cells which are carried there in the circulating current.

In this manner the multiplication of blood cells is shifted from place to place and becomes more and more localized until ultimately in higher animals the red bone marrow is the only body tissue in which these open spaces furnishing the required environment exist. It is, therefore, the only body tissue in which erythroblasts live and continue to multiply and give rise to the entire stock of red blood corpuscles which circulate throughout the body.

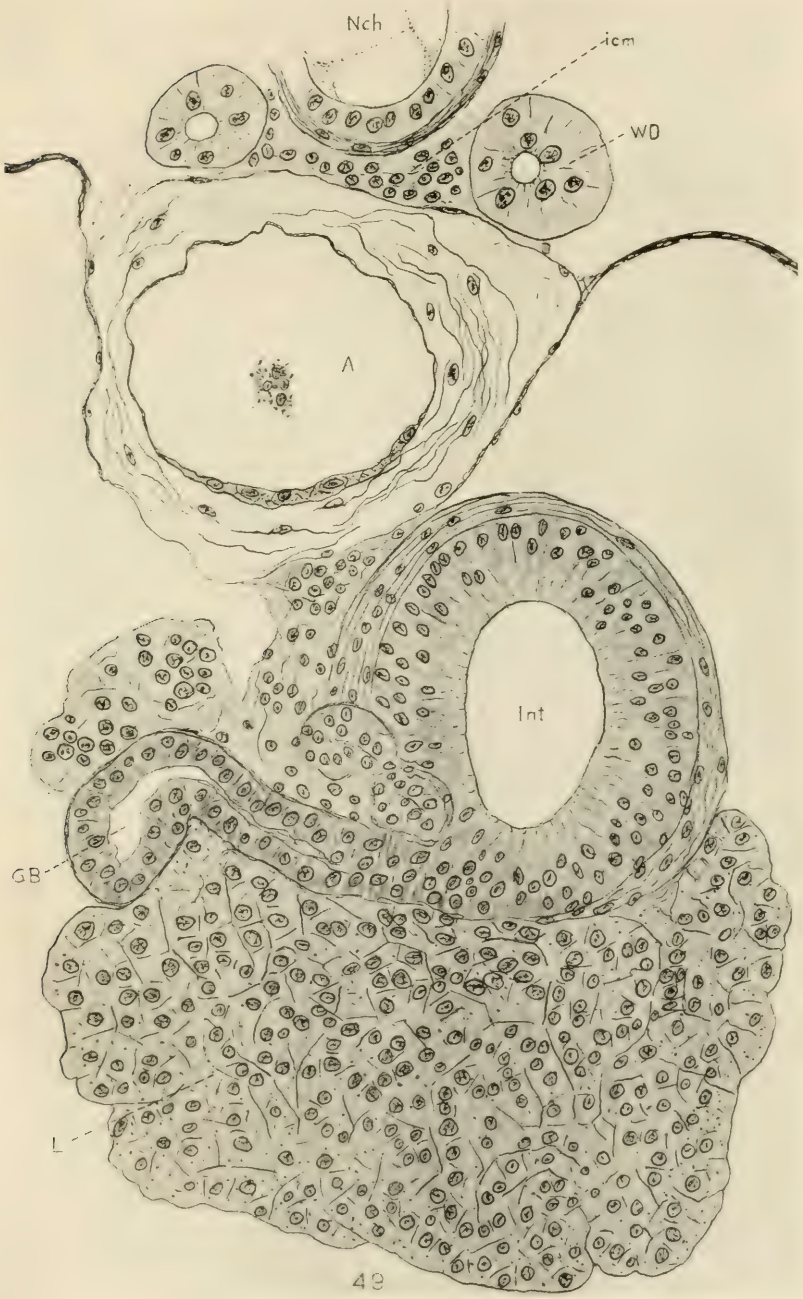
Blood cells always multiply in the unlined spaces but normally never multiply to any extent within closed vessels. It might be possible that certain abnormal growth tendencies on the part of the endothelium of the vessels and sinusoids in the bone marrow might cause an inclusion or vascularization of the spaces in which blood cells multiply and this growth might indirectly result in the cessation of the production of red blood corpuscles. This might be experimentally tested should some method be devised by which the growth of vascular endothelium could be so stimulated as to close the spaces of the bone marrow.

8. Question of haematopoetic organs

In the fish embryo the haematopoetic function of the liver is not of great importance. Yet in the liver of normal individuals numbers of blood cells are always found and numerous dividing blood cells are present. In the non-circulating embryos the blood is unable to reach the liver and in such cases there are no blood cells of any type to be seen in this organ.

Figure 49 represents a section through the gall bladder, bile duct and body of the liver in a sixteen-day old embryo. In

Fig. 49 A section through the liver of a sixteen-day-old embryo, without a circulation; Embryo 413, 1913. The gall-bladder, *GB*, and bile duct are seen connected with the intestine, *Int;* the liver, *L*, is a compact mass containing neither vessels nor any type of blood cell. *A*, the well developed dorsal aorta is lined by endothelium but its lumen is completely empty except for a slight coagulum near the center; *icm*, if followed posteriorly leads into the remains of the intermediate cell mass; *WD*., nephric duct; *Nch*, notocord.


Nch
icm
WD
A
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GB
L
49

this individual the heart is a solid string and the blood had never circulated. The liver presents a dense appearance, no blood vessels are seen and blood corpuscles are entirely absent. The general differentiation and condition of the tissues are, however, fairly normal and not at all degenerate. The intestinal epithelium is typical in structure. Above the intestine the well differentiated dorsal aorta is shown with connective tissue fibers abundantly present in its wall and a definite endothelial lining. The lumen of this aorta, however, has never contained any type of blood cells and the only solid particles within it are a slight coagulum near the center of the vessel. Above the dorsal aorta are the two Wolffian ducts and between them under the notochord are a few mesenchymal cells which represent more posteriorly the remains of the intermediate cell mass. Almost all of the erythrocytes in this mass have completely degenerated or have been destroyed by mesenchymal cells.

The embryos without a circulation thus furnish a definite means of establishing the actual haematopoetic value of any organ. They demonstrate that unless the blood current reaches the organ and thereby introduces embryonic blood cells into it the organ iself is incapable of giving rise to blood cells.

This experiment also demonstrates with equal force the inability of vascular endothelium to form blood cells in the fish. I can see no reason if vascular endothelium possesses a blood forming power why the aorta and other interior vessels of these embryos are invariably empty and never contain any type of blood cell. It cannot reasonably be claimed that this inability is due to the abnormal condition of the embryo having taken away the power of the endothelium to form blood cells, since it is so absolutely demonstrated that real blood forming material in other portions of the embryo possesses its perfectly normal capacity to produce blood and does produce it in a very abundant fashion.

These embryos furnish no evidence to indicate that there is any connection or association between the mesenchymal cells which are to form the connective tissue and those destined to form blood cells. There is no instance of a tendency for connective tissue cells to change into blood cells or of blood cells to give rise to any type of connective tissue cells.

Finally, one may conclude that the blood cells like many other specific tissues and organs have a definite localized specific anlage and that this anlage is distinct and separate in most cases from that of the vessel linings. In some cases, however, the blood and endothelial anlagen may come into intimate association, yet even here the two are probably of different mesenchymal origins.

A CONSIDERATION OF THE EXPERIMENTAL STUDY ON THE ORIGIN OF BLOOD IN TELEOSTS IN RELATION TO THE MORE RECENT STUDIES ON THE ORIGIN AND DEVELOPMENT OF VESSELS AND BLOOD CELLS

1. Introduction

This experimental study of the origin and development of blood and vessels relates itself to three more or less separate fields of investigation.

In the first place, the manner in which the blood anlage in Teleosts has separated itself as a unique intermediate cell mass has caused it to be studied as a special subject somewhat isolated from the more general literature on the development of blood in other vertebrates. Yet one very soon appreciates the mistake of this isolation since contributions such as those of Felix ('97) and Swaen and Brachet ('99, '01, '04), in particular, on the Teleosts are of more general importance than most investigations dealing with the broad subject of blood development in the vertebrates. The very fact that in this group the blood anlage is so peculiarly localized in the embryo lends itself as a great aid to the solutions of many questions of haematopoesis or blood genesis.

Secondly, a consideration of the origin and formation of the heart lining or endocardium and the vascular endothelium, in these embryos which have developed without having had plasma or fluids to circulate within their vessels, may furnish much important data towards a final solution of the origin and significance of endothelial lining cells, and the manner of spread and distribution of such cells through the embryonic body and the yolk-sac.

Lastly, such an experimental study bears closely upon the general questions of relationship between different blood cell types. The time and place of origin of the different cells, and the developmental relationship and powers of transmutability existing between various sorts of blood corpuscles as well as the endothelial lining cells of the vessel walls are all problems upon which the experimental results discussed above may throw light.

Each of these three divisions of the problem embraces an extensive and often cumbersome literature which it would be quite out of place to consider in detail at the present time. We shall, therefore, only consider the bearing of the facts recorded in the previous pages upon the opinions and positions maintained by the more recent investigators of the origin and development of blood and endothelium.

2. The specific problems of blood and vessel formation in the bony fish

It may be well to review first the special problems and questions involved in the development of the blood in Teleosts as a group. According to Swaen and Brachet ('01), the mesoblast in the middle and posterior regions of the trout embryo is arranged in two parts, a median primary somite portion and an outer primary lateral plate part. The lateral plate in the mid-body region then divides off a portion immediately adjacent to the somites to constitute the intermediate cell mass. Lateral to this a second part of the lateral plate is separated off to form the primary nephric duct. In the mid-region of the body the somites become separated from the primary lateral plate and the lateral plate pushes or grows towards the median plane and gives off a keel shaped mass between the somites and hypoblast. This mass unites in the median plane with a similar mass from the other side and here forms a large cell group triangular in cross-section, the intermediate cell mass. In the posterior region of the embryo a similar mass pinches away from the primary lateral plate and becomes the posterior continuation of the intermediate cell mass.

Anterior of the first somite in the unsegmented mesoblast of the head this division or pinching away also takes place. Thus the intermediate cell mass of the body becomes continuous with a definite lamella of the head. This well defined topographical portion of the embryonic mesoblast, the intermediate cell mass and cell lamella, is, according to Swaen and Brachet, the only material which gives rise to the heart, the chief vessels and the blood in the embryo. This description by Swaen and Brachet ('01) agrees very closely with that formerly given by Felix ('97), except that Felix disagrees in not deriving the aorta from the intermediate cell mass but from the sclerotoms.

The observations made upon the intermediate cell mass in Fundulus are in close accord with this summary. But no attempt has been made to solve the detailed question as to whether the aorta is derived from the intermediate mass or from the sclerotoms. It would seem that this vessel might arise from either source and still be formed from practically identical cells. Since in the separation of the primary lateral plate from the somite it is easily conceivable that some cells which generally accompany the primary lateral plate might be left as part of the lateral portion of the somite. This lateral portion of the somite is the part which later separates as the sclerotom so that the cells destined to form the aortic endothelium might occur equally well within the intermediate cell mass or within the sclerotom. Their location might vary among different species or even among individuals, and yet these aortic cells would be derived from the same genetic source.

Swaen and Brachet also indicate the head mesoblast as separated into three portions: the intermediate cell mass close to the top of the pharynx, the lateral plate split into two lamellae and the general head mesoblast close around the brain. The intermediate cell mass is more intimately connected with the splanchnic layer of the lateral plate. The pharynx widens in forming the gill pouches which continue to grow dorsally and finally separate the intermediate cell mass into two portions, one part thus comes to lie ventral of the pharynx and the other part dorsal. The ventral portions, at first solid masses below

either side of the pharynx, begin to migrate towards the middle line. The two masses fuse into one, spaces are developed in the mass and finally the endothelial lining of the heart is differentiated out of this group of cells. The lamellae of the side plate become separated and the space between them gives rise to the pericardial cavity. Oellacher ('73), Wenckebach ('86), Henneguy ('88), and Sobotta ('94) have all described the origin of the heart in Teleosts in much the same way.

Several of these investigators, Wenckebach, Swaen and Brachet and others, have called attention to a small mass of cells derived from the heart anlage which comes to lie beneath and outside the heart endothelium. This mass of cells has been claimed to wander away from below the pericardium and later to give rise to vessels and blood on the yolk-sac. In the non-circulating Fundulus embryos, however, neither vessels nor blood are formed on the extreme anterior portions of the yolk-sac. I have seen nothing in my studies which would indicate that any cells left over from the heart formation had wandered upon the yolk or given rise to blood cells or vascular endothelium.

Swaen and Brachet are alone in showing that the heart cells are definitely continuous with the intermediate cell mass of the the trunk mesoderm.

Many early workers on the fish embryo have claimed, as has been done for most vertebrae classes, that the heart lining arises from endoderm. The weight of evidence at the present time is so overwhelmingly against such a view that it warrants only a passing mention. Again, however, it must be realized that in the separation of the mesoderm from the endoderm it is possible that some future mesoderm cells may be left behind not cleanly separated. These cells might later isolate themselves from the endoderm to form vessels or blood. It nevertheless seems generally true that all blood forming cells are at one time in development contained within the mesodermal portion of the embryo. Gregory ('02) came to the conclusion that the endoderm and mesoderm could be traced to an indifferent cell mass mesentoderm in certain Teleosts, and according to his view, there is no way to say from which germ layer the heart endothe-

lium actually arises. A mixture of endoderm and mesoderm cells gave rise to endocardium.

The later development of the heart of the bony fish proceeds much as in the case of other vertebrates, as has been carefully described in detail by Senior ('09). The only point of interest in the present discussion is the origin and significance of its endothelial portions, and here Senior after a very thorough investigation confirms in all general points the previous findings of Swaen and Brachet.

In Fundulus as in other Teleosts the heart endothelium partially forms in loco but is also added to by wandering cells or ingrowths of mesenchymal cells adjacently located. The venous end of the heart leads directly down upon the yolk periblast, and as was shown in several figures, this periblastic material with huge amorphous nuclei may be at times drawn up into the cavity of the heart. This would indicate that the venous end was entirely free or not connected with any other vascular endothelium. This condition is, no doubt, due to the absence of the anterior yolk vessels which should in ordinary cases unite or fuse with the end of the heart so as to establish a closed circulation.

According to Swaen and Brachet in the region of the third somite the intermediate cell mass forms only the aorta, while caudad the aorta arises from the dorsal cells of the mass and the great part of the mass forms the red blood corpuscles and the venae cardinales. The endothelium of the cardinal veins finally surrounds the blood cells, but before these cells are fully developed or free, plasma has begun to flow in the aorta and other arteries.

In pelagic forms in which the egg is extremely small and develops very rapidly, the intermediate cell mass in the forward body sections is very small, sometimes only seen between the somites. This portion gives rise to the aorta. The cells are somewhat more numerous in the middle and posterior sections, but they never form a mass to the extent found in the larger demersal eggs. At the time of hatching the posterior cell strings form two lateral longitudinal vessels from the beginning of the mesonephros caudad to the anus. These two vessels, Swaen and Brachet consider to be homologous to the unpaired median stem vein

of the trout and this is thought to represent the conjoined cardinal veins. We have noticed that in Fundulus the intermediate cell mass is sometimes divided forming two lateral cardinals loaded with blood cells, while generally it exhibits a median unpaired condition. In the pelagic forms the vessels are all hollow at the time of hatching and the blood cells have not appeared.

Derjugin ('02) claims from a study of the pelagic egg of Lophius that the vessel cells of the aorta and cardinals are derived from the sclerotom. Felix ('97) like Ziegler ('87) differs with Swaen and Brachet ('01, '04) in that he derives the aorta not from the "Venenstrang" but from the sclerotom which under the notochord forms a mesenchymal aortic string. Felix states that no blood cells are to be seen in the aortic anlage, while the cardinal veins, of course, are loaded with the blood cells of the intermediate cell mass. Felix, therefore, derives the two chief vessels of the embryo from two different parts of the mesoderm, the somites or sclerotoms and the lateral plates.

Sobotta ('02) terms the intermediate cell mass "Blutstränge" and derives it from the lateral plate, though he had earlier claimed it to arise from the somites. He described it in the trout embryo in the region from the eighth to the thirty-third somite. The 'Blutstränge' at first paired, are naked cellular strings without a true vessel covering. This they receive later as the cardinal vein anlagen. The endothelial cells of the cardinal veins he derives from the same source which produces the aorta, namely, the sclerotoms.

Finally, then, Swaen and Brachet derive the blood and vascular endothelium of the aorta and venae cardinales from the intermediate cell mass which arises from the lateral plate. Felix derives only the blood and vascular endothelium of the cardinals from the intermediate cell mass which is separated originally from the lateral plate. The aortic endothelium arises from the sclerotoms. Sobotta considers the intermediate cell mass an exclusive blood forming material, while all vascular endothelium, including the heart, is derived from the sclerotoms which are budded off from the somite system. This disagreement, as we have pointed out before, is not of primary importance and

may result merely from the fact of the intimate connection of the sclerotom and intermediate cell mass before their original separation.

The question now arises whether all the blood of the Teleost embryo is exclusively derived from the intermediate 'Blutstränge.' Felix admits that the endothelium of the glomerular vessels of the mesonephros arise in loco and at the same time blood corpuscles often occur in this region. Sobotta claims that in the vascular network in the tail of the trout embryo some of the blood corpuscle anlage exists.

Both of these exotic positions of origin may be easily understood. In the first place, the nephric anlage is formed from cells in direct association with those constituting the early intermediate cell mass, and in the separation it probably happens that some future blood cells are held within the kidney anlage and these cells later develop in their proper fashion. The presence of blood corpuscles in the vascular network of the tail is due to the fact that the intermediate cell mass in many Teleosts, as Marcus ('05) has pointed out and as Senior ('09) particularly emphasized extends far back into the caudal region.

A similar consideration is the question of origin of vessels from material other than that of the intermediate cell mass and sclerotom. This is also important, and numerous observations would indicate that in the early bony fish embryo vessels unquestionably arise in loco and not solely as outgrowths or sprouts from a central vessel anlage. Sobotta ('02) on the contrary imagines a gradual growing away of the vascular system from its local origin, the sclerotom. The aorta is the primary vessel and, for example, the sub-intestinal vein arises from the aorta by vascular sprouts which grow around the gut, broaden out and fuse on its ventral side and finally give rise to the longitudinal vein. This theory of Sobotta is as unacceptable in the face of the great body of evidence to the contrary, Felix ('97), Rückert ('88), Hahn ('09) and many others, as is the opposite ingrowth parablast theory of His ('75).

The consideration has been confined so far to the intra-embryonic blood vessels. We may now briefly discuss the develop-

ment of vessels and blood upon the yolk. There are here two opposed or different views. The first derives the yolk vessels and blood directly from the yolk syncytium or periblast. The second derives blood cells exclusively from the intermediate cell mass in the embryo, but admits that cells may secondarily come to lie on the yolk by being pushed out from the intermediate cell mass with which, however, they maintain a definite continuity. The vascular endothelial cells are also derived from the embryo as mesoblastic wandering cells, but these are not to be compared directly with blood cells since their parent cells have a separate place of origin. Most of the earlier workers thought that the blood in the Teleosts arose on the yolk-sac, as it does in other meroblastic embryos. The more recent workers have gone to the other extreme and deny the presence of blood islands upon the yolk-sac as separate from the intermediate cell mass.

As mentioned in describing the heart formation, numerous investigators have recorded wandering mesenchymal cells upon the yolk-sac, but from a study of the literature no clear conception can be formed as to the origin of blood cells or the vascular endothelial cells upon the yolk from these wandering cells. Some authors claim that the majority of wandering cells become pigment cells, while the remainder form the yolk vessels. In Fundulus the pigment cells very soon present a different appearance from the mesenchymal cells which are to form the vascular endothelium. Both types of cells may be readily seen wandering over the yolk between the ectoderm and periblast. Before the yolk vessels are completely formed, the circulation of a cell free plasma has begun. The extent of the spaces in which this circulation takes place is very variable. The arrangement of the veins of the yolk circulation is also extremely different in the different groups of Teleosts. One must agree with Hochstetter ('93) in stating that the yolk circulation in different forms is so different from the start that it is not possible clearly to summarize the condition in order to give satisfactory comparisons with the same vessels in Selachians, and Amphibians.

When the plasma is flowing in a closed system within the embryo, it is still running as a wandering stream through lacunae and sinuses on the yolk. This probably explains why the blood cells reproduce for so long a time on the yolk-sac while no such reproduction is taking place in the well formed vessels of the embryo.

It is difficult to determine the exact moment, when, or place at which the first blood cells get into the circulation. This probably varies even among embryos of the same species. Ziegler ('88) thinks, however, that just beyond the lateral plates in the plasma filled spaces of the yolk-sac which lie between the periblast and ectoderm, the first blood cells project into the circulation. They are in the form of cell strings which later connect the cardinal veins with the vascular yolk net. Swaen and Brachet saw in trout embryos of eleven days in the region of the fourteenth somite and posterior that the intermediate cell mass spreads out laterally below the lateral plate and on to the yolk surface. The cells thus came to lie above the yolk syncytium and first attained their red color in this position. These authors thus claim that in the bony fish with a large yolk-sac the haemoglobin free early blood cells through continued contact with the yolk become transformed into erythrocytes.

The experimental embryos considered in the present paper demonstrate, however, that it is not at all necessary in such a Teleost to have the erythroblasts reach the yolk-sac in order to acquire their red haemoblobin condition. The tightly packed erythroblasts within the intermediate cell mass of the embryo develop perfectly and readily attain a normal red haemoglobin color.

Finally, comparing the processes of vessel and blood formation in Teleosts with these processes in other vertebrate embryos, we find no definite explanation for the formation of the intermediate cell mass. In other embryos the blood is largely formed upon the yolk. However, it must be recognized from recent contributions that the formation of intra-embryonal blood is much more extensive and important than has formerly been

supposed. The relation of the blood anlage to the cardinal vein and the position of the blood forming cells dorsal of the gut are unique in the Teleosts. The late formation of the yolk vessels and their type of origin from wandering mesenchymal cells is also of special interest.

It would seem as though the peripheral mesoblast which in other vertebrate types grows and develops outside the embryo, had in the Teleosts been peculiarly concentrated and drawn into the embryo during its phylogenetic history. Yet in this intra-embryonic position the peripheral mesoblast gives rise to the same cells which it would ordinarily produce on the yolk-sac. The different Teleosts probably show this drawing in of the peripheral mesoderm to various degrees so that in some cases only part of the mesoderm is incorporated in the intermediate cell mass, while the remaining part may still be outspread upon the yolk and there differentiates extra-embryonically. The intermediate cell mass is connected caudally with the end bud, just as the peripheral mesoblast of the Selachians is with the blastopore lip. In its genesis the intermediate cell mass is split off from the lateral plate and localized along its median border.

Marcus ('05) in his study on Gobius capito advanced the opinion that the intermediate cell mass in this embryo is comparable to the peripheral blood forming mesoderm of other meroblastic eggs. In an embryo of eleven somites, the intermediate cell mass passes without a break caudad to the end bud and there connects with both the ectoderm and entoderm, just as the peripheral mesoderm would meet the other two germ layers at the blastopore lip. He attempted to show by diagrams the relationship between the intermediate cell mass in Teleosts and the blood forming mesoderm of Selachians.

As the homologue of the peripheral mesoderm the intermediate cell mass has the power to form vessels and blood cells. Most authors admit this power and only Sobotta ('02) denies the vessel forming property, while others claim that only the cardinal veins arise from the intermediate cell mass, still others, as Swaen

and Brachet, would derive the endocardium and aorta also from this common source.

The important fact is that in the small pelagic embryos, where no blood formation takes place before hatching, the intermediate cell mass forms the aorta and the cardinal veins and is also derived from the lateral plate. The lateral plate thus contains cells capable of forming vascular endothelium, and this is the case in all vertebrates.

At an early time in evolution the extra-embryonic blood forming mesoderm has been included within the body of the Teleost embryo and lies over the gut as the intermediate cell mass representing the yolk vascular layer. Here it is important to note that the yolk-sac of the Teleosts contains no real mesodermal layer, only separate wandering mesenchymal cells are found between the ectoderm and periblast, and these wandering cells have migrated out from the embryo.

Finally, as Mollier ('06) states in his review of this subject, it is not a question of the formation of the intermediate cell mass in the individual bony fish, but the wider question of the behavior of the blood forming peripheral mesoderm in the bony fish. All of the results must be considered in this light in their application to other animal classes.

The intra-embryonic blood formation in the bony fish does not represent the primitive type for vertebrates as Sobotta ('02) claims, but this is, no doubt, a modified secondary condition accompanying the various other modified and special developmental processes which bony fish embryo so frequently presents.

Wilson ('91) states of the mesoderm of the Teleost that: "The ventral subvitelline mesoderm, having in this way losts it function in the Teleost, must be regarded as a rudimentary organ of the gastrula. It always remains very small, and does not form any special organ or set of organs in the embryo." The real fact is that the subvitelline mesoderm is misplaced, being within the embryo as the intermediate cell mass and here forms the blood of the individual and, therefore, the yolk-sac of the bony fish has no mesodermic layer.

3. Vascular endothelium, and vascular growth and development

Mollier ('06) concludes in his review regarding the origin of vessels as follows.

> As to the genesis of embryonal vessels we may pass the judgment that the theory of the local origin of the vascular endothelium is valuable. The notion of His ('75) and Vialleton ('92) that the vessel strands of the embryo grow in as sprouts from the extra-embryonal anlage (vascular anlage) is not nearly so probable as that the individual vessel cells arise in loco and thus form the vascular nets.

This statement agrees in every way with the contentions so fully presented by Huntington ('10, '14), McClure ('10, '12) and others, regarding the origin of lymph vessels. Lately it receives additional substantiation from the experimental results recorded by Miller and McWhorter ('14) on the origin of blood vessels in the chick embryo. Such a position is further strengthened by the still more recent experimental evidence, presented by Reagan ('15) which shows the origin in loco of vessels in isolated parts of chick embryos. All of these experiments confirm the earlier results of Hahn ('09) on the origin of vessels in the chick.

In the Teleost embryos studied during the present investigation there can be no doubt that the heart endothelium and aortae arise in loco within the embryo, and here there are no vessels, or even mesoderm, present on the yolk-sac in the anterior portion. Certain vessels do partially grow from the embryo out on to the yolk-sac and other smaller vessels arise in many separate regions of the yolk-sac as the products of wandering mesenchyme cells which become arranged to form the tubular vessels. All of these vessels after they have arisen may grow by budding or sprouting off new vessels or may increase in length by a forward growth so well described in living embryos by E. R. Clark ('09, '12) in his careful studies of this subject.

Felix ('97) describes the origin of the aorta as follows:

> The 'mesenchymaortenstrang' arises from the two lines of sclerotoms after they are finally pinched away from the somites. No fusion of cell material occurs between this and the 'venenstrang,' the intermediate cell mass. This 'mesenchymaortenstrang' comes from that

part of the somites that was immediately in contact with the intermediate cell mass portion of the primary seitenplatte. As the forward somites bud off sclerotoms, these also are added to the 'mesenchymaortenstrang.'

The median part of the 'strang' forms the aorta, 'aortenstrang,' the lateral the 'mesenchymgewebe' (mesenchymestrang). *The 'aortenstrang' is at first solid and does not obtain a continuous lumen to begin with, but here and there develops a space, and these spaces become confluent to form the tubes and build the paired aortae.* Certain portions of the strang remain solid much longer than others. The association of the paired aortae to form an unpaired single vessel soon follows. *While the aorta is being so formed, one never finds blood cells within its lumen.* Blood cells occur only in the 'venenstrang' and in certain vessels of the nephric glomeruli. Occasionally certain of the glomerular vessels contain blood corpuscles at a time when the blood circulation is not yet closed.

Felix ('97) cites the observation of P. Mayer ('94) on very young Selachian embryos in which the medulary tube was still open. It was found in such embryos that the aorta is segmental and derived from the somites and subsequently the longitudinal tube is formed by the fusion of these isolated points. Felix agrees with P. Meyer's observations from his study on the Teleost.

There has been great diversity of opinion regarding the germ layer from which the vascular endothelium and blood corpuscles arise. In the literature it may be found that certain competent investigators have in each vertebrate class claimed the vascular endothelium and blood cells to be derived from the endoderm, while other workers of equal authority have found the vessels and corpuscles to arise from the mesoderm. The consistency of the disagreement which one finds in a review of this literature is most peculiar. These disagreements have their foundation in the extreme difficulty of the problem on fixed material.

It is interesting to note that in no case has the same author derived the blood and vessel endothelium from different germ layers. Each author always takes the position that blood and vascular endothelium arise from either the mesoderm or the endoderm.

We have here much to do with wandering cells which become lost from their epithelial layer, and it is impossible to state their

origin. This is left to the imagination of the individual investigator and further possibilities of error are open.

Wenckebach's ('86) observations of living embryos are most important in this connection. He noted that not only the layers but that independent mesoblast cells with amoeboid processes wander out of the embryo and over the yolk. These wandering cells play a great part in the formation of the anlage of the heart endothelium and great vessels. In the Teleost embryo one may readily observe these wandering cells in the yolk-sac, and they doubtless give rise to the yolk vessels and blood islands as well as the pigment cells so abundantly present.

Ziegler ('87) has suggested that it may be that the blood anlage in phylogeny has been passed to the mesoderm from the endoderm, and for this reason the endodermal origin may sometimes occur in coenogenetic development. Goette ('90) also held that the endodermal origin of the blood was the more primitive one. This point of view overlooks the fact that in the invertebrates generally the blood and vessel walls are derived from the mesoderm.

In discussing the question of the place of origin of the vessels, Felix ('97) points out that Rückert ('88) claimed in Selachians, that the aorta arose *in loco*. P. Mayer and Strahl ('95), have also stated that the great vessels are late in appearing and arise *in loco* in the embryo's body. Felix states that the glomerulus of the bird mesonephros originates *in loco* independently of the aorta. Further that the stammvene, venenplexus of the mesonephros, certain vessels of the glomerulus, and also the mesenteric artery along with the aorta in the Salmoniden arise *in loco*. Regarding the anlage of the heart and vena sub-intestinales, Felix is not certain but thinks that these likewise arise *in loco*. All of these observations are directly opposed to the theory of ingrowth of vessels from the yolk-sac, the parablast theory of His ('75) as well as the outgrowth of vessels in the sense advocated by Sobotta ('02).

Ziegler ('89) and Felix ('97) have both speculated considerably as to the relationship of the cavity of the circulatory system with the primary body cavity and the coelom. Ziegler pointed out

that in the phylogenetic origin of the blood vascular system we have the following changes: The primitive condition is represented by the development of a space between the body wall, the ectoderm, and the gut wall, the endoderm, that is, the primary body cavity or protocoel. Embryologically the blastocoel of the blastular or after gastrulation, the space between the invaginated endoderm and the ectoderm, the schizocoel, represents the primitive vascular space. The body cavity in rotifers, nematodes, bryozoa and arthropods is a primary body cavity and is filled with a fluid, the haemolymph. In the arthropods on the dorsal side of the body is the pulsating heart which sets the fluid in circulation and this fluid contains corpuscles similar to the white blood corpuscles of vertebrates.

In the arthropods the vessels and heart are often highly developed but all communicate with lacunae and spaces between the gut wall and body wall. The heart is surrounded by a pericardial space (not truly coelomic) which is full of haemolymph, and as the heart pulsates this haemolymph is drawn in through ostia along its walls and then propelled out through the aorta and its arches to the vessels and spaces of the body. These body spaces, or the haemocoele, are thought by some to be a secondary or specialized cavity. Yet it is not coelomic and has no definite lining and resembles very closely the primary body cavity of the rotifers, nematodes, and other invertebrate forms which it most probably represents. In some of the higher Crustacea a secondary body cavity or coelomic space of limited extent is present enclosing the ophthalmic artery in Paelamonetes. The cavities surrounding the gonads are also coelomic, and since these are well developed species the coelomic space here probably represents a progressive rather than a regressive condition.

The second step in Ziegler's evolution of blood vessels is illustrated by the conditions in the molluses. In these animals between the gut and body wall lacunae and interstitial spaces exist which occupy the position of the primary body cavity and these are filled with blood. Vessels lead into the lacunae and the cavities of these vessels as well as the cavity of the well formed heart are also considered to be part of the primary body

cavity with which they are continuous. The pericardial cavity in the molluscs is true coelom and not a part of the primary body cavity and contains no blood. In almost all of the molluscs the pericardium is in communication with the nephridia and the nephric duct usually leads from the pericardium to the outer body-wall. The pericardial cavity in contrast to the primary body cavity is designated as secondary body cavity or true coelom.

The final step in the phylogeny of the blood vascular system is characterized by an important expansion of the secondary body cavity or coelom as is the case in the echinoderms, annelids and vertebrates. As a result of the expansion of the secondary body cavity, the primary cavity is reduced merely to a system of channels or vessels and small interstitial lacunae. In the vertebrates, therefore, according to Ziegler, the blood and lymph vascular system represents the persistent part of the primary body cavity. Ziegler considers the blood vascular system and lymph vascular system to have had a common origin. The blood vessel endothelium is closely similar in all respects to the lymphatic endothelium. He thus agrees with Bütschli ('82) that in all metazoa the blood vascular system has its origin from the blastocoel.

Felix ('97) holds that his studies on the Salmoniden will not fit into Ziegler's scheme. He claims that the origin of the stammvene in the cranial portion is the same as that of the primary mesenephros in the caudal region, and is also of the same origin as that of the primary nephric duct. Cells of the splanchnic as well as cells of the parietal layer of the mesoderm enter into the structural material of the stammvene. The cavity of the venenstrang is the same as the cavity between the lamellae of the secondary lateral plate, that is, true coelomic cavity. The three structures referred to are all portions of the same base, the lateral plate mesoderm, the primary seitenplatte. Felix states, as there is little doubt that the cavity of the primary nephric duct is homologous with the secondary body cavity, so there is little doubt that the cavity of the venenstrang is also. The development of the aorta shows similar relations. It arises, according to Felix, from the sclerotomes which come from the

somites and contains both the somatic and splanchnic layers of mesoderm. The origin of the aorta from the 'mesenchymaortenstrang' is from the same cell material as the mesodermal layers. The cavity of the myotom is secondary body cavity, coelom, and so also is the aortic cavity. Neither is in any way primary body cavity. The formation of the aortic cavity is a similar process to the canalization of the stammvene. Felix in this way arrives at a conclusion diametrically opposed to Ziegler.

These conclusions he recognizes are not facts but are based on facts obtained from a study of Teleosts which are a side branch of the vertebrate stem, but from which one may still generalize to some extent. Felix calls attention to the fact that in the selachians Zeigler ('92), and in the reptiles Strahl ('83, '85), and in the birds Kölliker ('84) and Ziegler ('92), and in the mammals Kölliker ('84), all claim that the first vessel anlagen are found in the mesoderm and not between the mesoderm and endoderm. Only in the mesoderm the secondary body cavity arises by splitting, and since the solid vascular anlagen are formed within the mesoderm their cavities should not be considered primary body cavity. The writer is entirely unable to agree with such an analysis of the origin of vessels, particularly yolk vessels, as well as of the primary and secondary body cavities for reasons given below.

Felix ('97) now goes further and assumes that the lymph vessels arise in mesenchyme and their cavity is primary body cavity and their wall cells are modified connective tissue cells. This position is difficult to appreciate since it must be admitted that mesenchyme is a direct product of the mesoderm, and, according to Felix, any definitely formed cavity arising between such cells would seem to be coelom. I question, however, whether any other morphologist would put the same interpretation on all the spaces cited by Felix as being in the coelomic category. Felix states, for example, that the aorta arises from a mesenchymaortenstrang derived from the sclerotom. The sclerotom is more or less mesenchymal in nature and certainly contains many cells which will later give rise to types of con-

nective tissue. If the aorta did arise from this group of cells its cavity is scarcely of an origin comparable to that of the coelom. Its endothelial wall is certainly much the same as that of the lymph vessels.

The cavities of the nephric duct, ovarian duct, kidney tubules and other tubules derived from the mesoderm are not usually considered to be parts of the coelomic cavity. The blood and lymph vessels do arise from the mesoderm but not in such a way that their cavity can be readily homologized with the coelomic space originating between the lamellae of the mesoderm. The vessels on the yolk-sac of the Teleosts are formed from disconnected wandering mesenchyme cells which are easily demonstrated. The cavity of these vessels surely cannot be interpreted to arise between mesenchyme cells some of which are derived from the somatic and some from the splanchnic mesodermal layers. The yolk vessels in Teleosts arise by arrangement of mesenchyme cells and so apparently do other vessels within the embryo. Thus these blood vessels are similar in origin to the lymphatics according to Felix's notion of the mesenchymal origin of lymphatics. The numerous recent investigators of the origin of the lymphatics, athough to some extent divided into two schools, all treat the lymph vessels and blood vessels as being of the same general genetic type Sabin ('13) and Huntington ('14).

Finally, the most damaging evidence against Felix's notion that the blood vascular spaces are derived from the coelom, and that these spaces are actually now comparable to the coelomic space is the following: Before a true coelom, such as that to which Felix refers in the vertebrates, has arisen in the animal series blood vessels are already present and these vessels often communicate with or are actually a part of the primary body cavity. When the true coelom does arise in the invertebrate series blood vessels never open into its cavity or communicate with it. Felix has therefore derived an older and more generalized animal system from a newer or later formation. This of course is contrary to any principle of phylogenetic calculations.

The weight of evidence at the present time is then in favor of the earlier notion of Ziegler. The blood vascular system if it is associated with, or phylogenetically derived from any other body cavity, that cavity is really the primary body cavity or embryologically the blastocoel.

4. Haematopoesis, the monophyletic and polyphyletic views, etc.

The experiments recorded above are of particular value in the solution of that very complex problem, the origin and relationship of the different types of blood corpuscles. We may here then briefly consider the evidence they furnish in connection with the various theories and points of view recently advanced in explanation of the origin of blood cells.

The vertebrate animals present two entirely different types of cells floating in their blood fluid. The white blood corpuscles are cells of primitive type and are not only found within the vessels but they also wander through the interstitial spaces of all the tissues of the body. These wandering white blood cells, amoebocytes, are almost universally distributed throughout the animal kingdom being found in all the invertebrate groups above the one or two very lowest as well as in all the vertebrate classes. In no animal do these cells contain haemoglobin, haemocyanin or any compound that would particularly qualify them as oxygen carriers, or give to them any function as an organ of respiration. These white blood cells found outside of the blood currents as well as in the blood are to be looked upon as cells which are not particularly associated with any specific blood function. They merely find the blood current a ready or rapid means of being carried from place to place within the body.

The red blood corpuscles, erythrocytes, are in contrast to the white cells a very highly specialized type of cell and specifically a blood cell. In fact, this is one of the most specialized cells within the body. In mammals, for example, it is specialized to such a degree that its functional perfection is actually accompanied by the loss of its nucleus and necessarily, therefore, the loss of its own future existence after a short period of time.

Contrasted with the almost universal distribution of the white cells within the animal kingdom the erythrocyte is confined to the vertebrates phylum and to certain particular cases among the invertebrates. The respiratory function of the invertebrate blood is often claimed to be confined to the fluid or plasma mass, and only among certain members of the higher groups is a cell developed with the function of carrying oxygen to the body tissues and even this cell can not be said to possess the regular typical characters of the vertebrate erythrocyte.

The vertebrate erythrocyte along with the typical vertebrate mouth, the pharyngeal gills, the dorsal nerve cord, the notochord, and bony skeleton and the many other possessions characterizing the vertebrate group, separates it in gulf-like fashion from the invertebrates. The white blood cells bridge this gulf but the red blood corpuscle differs from that of the invertebrate in a way comparable to the difference between the vertebrate mouth and that of the invertebrate, both serve the same function but are structurally unlike. Just as the mouth and pharyngeal gills and vertebral column have no invertebrate forerunner, so no cell within the invertebrate animals can at the present moment be sought out or designated as the certain ancestor of the red blood cell.

The cells of the vascular walls are closely similar in both vertebrates and invertebrates, as pointed out above. In both animal divisions they probably arise and develop in the same fashion. The white blood corpuscles probably do also. Yet the red cells, although they too originate from the mesenchyme in the vertebrates, are not in any way certainly descended from the invertebrate oxygen carrying cell or the wandering leucoblast or amoebocyte.

There is certainly no phylogenetic or comparative morphological evidence to warrant one in deriving vascular endothelium, leucocytes, and erythrocytes from a common cell ancestry except, of course, they are all derived from the mesenchyme or same germ-layer.

The fundamental histological study of the early developmental stages of the blood elements in vertebrates was contributed by

Van der Stricht ('94). His studies were especially confined to the mammals. As has often been the case the conclusions reached from this pioneer study are largely correct in the light of recent investigations. Van der Stricht held that the first blood cells arising within the area vasculosa are entirely young red cells, erythroblasts. When one surveys the literature of this subject, it is found that all authors with three or four recent exceptions (Bryce ('05), Dantschakoff ('07) and Maximow '09), hold that the blood islands give rise exclusively to red, haemoglobin bearing corpuscles, erythroblasts or finally erythrocytes. This is true for the Fundulus embryos described in this paper and even though the cells are confined to their place of origin and never flow away, since there is no circulation, yet the groups always consistently contain only erythrocytes.

Van der Stricht holds that the leycoblasts and leucocytes are independent of the erythroblasts and arise extra-vascularly in the mesenchyme and later wander into the vessels.

Browning ('05) and Goodall ('07) have both recently claimed that the leucocytes have a different origin from the erythrocytes and arise at a later period. Goodall states:

> When leucocyte proliferation in the liver has begun, the islands of erythroblasts and leucoblasts are definitely separate in position, and the distinctness of their identity is obvious, and no transitions between them can be seen. These facts argue strongly against the view that the erythroblasts are derived from the primitive leucoblasts.

Jolly and Acuna ('05) have pointed out that in early stages only red cells are found in the blood. The first lymphocytes occur very late and still later the granulocytes, so that the guinea-pig embryo has attained a length of sixteen mm. before white blood corpuscles are present.

Again all authors with few exceptions seem entirely agreed that the leucoblasts arise much later than the erythroblast. All without exception also agree that the leucoblasts arise extra-vascularly while the erythroblasts arise partially within the sinuses, and that the island groups of erythroblasts soon become surrounded by vascular endothelium while no vessel walls have ever been described to form around the groups of leucoblasts. These facts are no doubt of much genetic importance.

The question involved is then: Which is correct, the monophyletic or polyphyletic theory of haematopoesis? It is recognized by all that both propositions are classed only as theory. It must further be recognized that both theories are based at the present time only upon the interpretations of various observers, these interpretations are not necessarily facts. I trust, therefore, that the experiments on Fundulus embryos may add a basis of unquestionable facts which may show the correctness of one or the other of these interpretations.

With this point of view, we may undertake a critical examination of the evidence so ably presented by Maximow ('09) in his study on the mammalian embryo. The observations he construes as strong argument in favor of the monophyletic origin of all types of blood cells and vascular endothelium. This contribution by Maximow ('09) has been accepted by many embryologists *à bras ouverts*, and has been largely incorporated into several recent chapters on the development of the blood, for example, by Schaefer ('12), and Minot ('12).

In the primitive streak stage of the rabbit embryo Maximow states that the peripheral mesoderm in which the blood islands will later occur has in no sense the character of a connected epithelial layer, but consists merely of local accumulations of cells of mesenchymatous type. The cells of this mass are of long thin spindle shape or with star-like processes. These cells are probably much of the same type as the wandering cells seen on the yolk-sac of the Fundulus embryos. In this peripheral mesenchymatous mesoblast the first blood islands arise in the caudal part of the area opaca, as originally described by Van der Stricht. The blood islands are formed from the spindle or branched mesenchyme cells which become associated into groups.

Maximow states that the first endothelial cells like the primary blood cells are also derived from the mesoblast-mesenchyme. With this one may fully agree and several other tissues could be included in the statement as derived from mesenchyme. Maximow, however, goes further and thinks this general source a common specific source. Thus the endothelial cells and blood cells are closely related and arise from a common stem cell in

the blood islands and may continue to arise from such a cell during later development.

Die ersten Endothelien und die ersten Blutzellen sind also beides Mesoblast- resp. Mesenchymzellen. In den Blutinseln sehen wir sie von unseren Augen aus einer gemeinsamen Quelle entstehen. Auch in der späteren Entwicklung werden wir oft Gelegenheit haben, die enge Verwandtschaft dieser beiden Arten von Mesenchymzellen zu beobachten.

This is merely a matter of interpretation and not at all a demonstrated fact. In reply to such a position we must call for an explanation of the demonstrated fact presented on previous pages showing that vascular endothelium forms in a perfectly normal fashion within the heart and head region of embryos without circulating blood, but in no case in early or late stages was the endothelial lining of the aorta or other vessels capable of giving rise to any type of blood corpuscles. Yet the power to form blood corpuscles was abundantly present in the same embryos as shown by the huge numbers of blood cells within the blood forming regions, the intermediate cell mass and yolk islands. Why do not the mesenchyme cells within the liver and all vascular endothelium form blood when no circulating blood reaches them? (If ever, there should then be the stimulus to give rise to its formation).

The red blood cell anlage is a definite mesenchyme cell or group of cells and only members of this cell group possess the blood forming power. To cite a parallel case, the liver cells are derived from the common endodermal cell stock yet not all early endodermal cells, in fact only a few, have the power to develop into a liver, or a pancreas or a lung as the case may be. The embryological argument is indeed rather loose that on account of the fact of vascular endothelium and blood cells arising from mesenchyme would assume, therefore, that these very different cells had a common stem mother cell and later actually possessed some powers of transmutability.

Maximow advances the interpretation that the first blood cells in the area vasculosa are not all erythroblast or future red blood corpuscles. These cells he designates as 'primitive blood

cells' since they may form either white or red corpuscles. Yet in the yolk islands of the Fundulus embryos without circulation only red blood cells, erythrocytes, are produced and they remain in this location to be observed throughout embryonic life. The evidence for Maximow's position seems to me somewhat insufficient.

During the summer of 1914, I had the privilege of examining Mme. Dantschakoff's preparations which both she and Maximow cite in support of the monophyletic theory of blood cell origin. One so inclined might interpret these specimens as showing that the red and white corpuscles do arise from the common stem mother cell. The youngest lymphocytes were invariably scattered among the mesenchymal cells while the erythroblasts were budded off into more or less well defined vessels. No one could emphatically state that the two classes of blood corpuscles had ever actually divided off from any one single mother cell.

The more or less constant separation of the early leucoblasts and erythroblasts, as is also shown in Maximow's figures and those of other workers, would seem to indicate their origins from two different mother cells. If one mother cell only forms or divides off cells which develop into lymphocytes or leucocytes and another mother cell gives rise to only erythroblasts, then there is no reason to say that the two mother cells were the same although they appeared to be two similar mesenchymal cells. They were potentially different, and this potential difference is all that the diphyletic notion of blood cell origin demands. A careful study of the embryos without a blood circulation will demonstrate the fact of this different origin of white and red corpuscles.

Maximow then advocates the last clause in the monophyletic code, and states that the intravascular primitive blood cells are not only increased by mitosis but are also added to by the production of the same kind of cells from the fixed endothelial wall of the primitive vessels. Endothelial cells may wander away into the mesenchyme or may wander into the vessel lumen. One often sees according to Maximow a cell project into the vessel, its body assumes a rounded form and its protoplasm

changes into that of an erythroblast. It must be distinctly remembered that these appearances are in dead stained specimens and many possibilities exist which might explain their occurrence.

Granting that such a phenomenon actually appears to occur there is one very probable explanation without assuming that true vascular endothelium may form blood corpuscles. Let it be supposed, for example, that in the formation of the vascular wall around the yolk-sac blood islands that some of the peripheral cells of the island might lag behind in their differentiation retaining their more or less mesenchymal type. Such a cell may come to be closely pressed against the vascular wall and really appear as though it were one of the vessel wall cells. This might readily happen, and probably does happen, and may account for the occasional appearance of 'vessel wall cell' forming a blood cell.

Why do not the endothelial cells in the experimental embryos possess the power to form blood cells when the vessel is totally empty of blood cells? Even though it is clearly shown that other cells of the embryo do possess the normal blood building power. These specimens are exactly such as should supply definite proof of blood cells arising from endothelium, but the evidence they furnish really disproves the proposition.

Schridde ('07, '08) according to Maximow has gone so far as to claim that in young human embryos endothelium can directly form primitive erythroblasts. Maximow does not agree with this since in his specimens the endothelium gives rise only to indifferent colorless cells. Shridde's claim is based upon misinterpretation and so, I believe, is any claim that blood cells arise from formed vascular endothelium.

Most authors find that in the very early embryonic blood there are no white corpuscles but only red cells present. Bryce ('05) however, describes in Lepidosiren the very early origin of leucoblasts from primitive blood cells, and later Dantschakoff and Maximow find lymphocytes not only in the vascular net of the area vasculosa but also, though at first very few, in the circulating blood. Maximow thinks that when these early

lymphocytes are not seen it is due to poor technique or defective material.

Maximow believes the red blood cells may finally arise from lymphoblasts as erythroblasts, then erythrocytes. This mode of development of the definite erythroblasts continues throughout life and is accomplished in the same manner in all erythropoetic organs. Wherever such indifferent mesenchyme cells, lymphoblasts, are found this locality is eo ipso a new place of origin of erythroblasts out of these colorless stem cells. If this be actually true, why then do not red cells, erythroblasts, finally form all through the body of non-circulating fish embryos, since the wandering lymphocytes surely have the power to reach many places other than the normal sites of erythroblasts formation, the intermediate cell mass and yolk islands?

Maximow claims that both types of blood cells red and white arise at one and the same period from one and the same source, the primitive blood cells in the area vasculosa. The experiments on Teleosts do not bear out such a position since the original or first blood cells from the intermediate cell mass are all erythroblasts and show a characteristic type at a very early time. The blood origin on the area vasculosa is not so extensive, but here also first form only erythroblasts.

Supporters of the polyphyletic origin of blood cells have been able to make equally strong observations in favor of their view on similar material to that studied by Maximow, Dantschakoff and other advocates of the monophyletic theory.

Maximow suggests that since the "primitive blood cell" has no haemoglobin it really stands nearer to the leucocyte than to the erythrocyte, and one might say that the leucocyte arises first in development and the haemoglobin cell later. This is most decidedly not the case in the Teleosts where the primitive mesenchymal blood cell passes directly into the erythroblast without ever showing a stage suggesting either lymphoblast or leucocyte.

With Weidenreich ('05), Maximow takes the position which he had earlier manitained that all non-granular leucocytes and also the wandering cells of the tissues constitute one great cell

group. The position determines the direction of their development and only in certain places does one find all types being formed, for example, in the embryonic liver and the adult bone marrow.

In reply to the extreme monophyletic position it may be asked: Why are only erythrocytes present in the old blood islands on the yolk of non-circulating specimens? Why is no cellular blood element present in the aorta and other endothelial lined vessels in the anterior region of similar embryos? Why are wandering "primitive blood cells" unable to form blood in the liver and other positions while blood forming power is present to a vigorous extent in certain regions of the same embryo but from a definite anlage? Wandering cells in the Teleost embryos have to do with yolk-sac blood origin, but these wandering cells are the equivalent of a part of the peripheral mesoderm and always wander out on to comparable regions of the yolk and never wander to other places within the embryo.

Maximow probably criticizes correctly the many artificial distinctions between various leucocytes which are pointed out by some advocates of the polyphyletic theory. My experiments do not bear on this point up to the present time.

Finally Maximow does not imply that a granular leucocyte or red corpuscle can change into anything else, they cannot undifferentiate. He states that before there is any development of granulation or haemoglobin in two different cells, there is really a difference in the cells though we are unable to distinguish it. This invisible difference determines the destiny of the cell to form either a leucocyte or erythrocyte. This is certainly true, but we cannot stop just at this point; these differences must be carried a step further or really back to their actual beginning. Then it is found that although two wandering mesenchyme cells on the yolk-sac of the fish embryo are indistinguishable so far as our powers of observation go, yet they are fundamentally different since one is destined to form only an erythroblast while the other possesses no such power and can form only an endothelial lining cell or pigment cell as the case may be.

This is all the diphyletic or polyphletic school would ask. That is, that certain definite mesenchymal cells are actually the red blood cell anlage and only from these particular mesenchymal cells do red blood cells arise. Here we logically stop, for this is what is conceived by embryologists to be an anlage, back of this we go to certain germ layers and still further back to the developmental potentialities of certain individual cells as followed in studies of cell-lineage and finally we reach the elementary proposition of deriving everything from the original egg cell. But to stop with the tissue anlage we find strong evidence to indicate that certain special mesenchymal cells are designated to form erythroblasts, others leucoblasts, and still others, and these are much more universally scattered throughout the embryo, give rise to vascular endothelium. These latter may really be admitted to form endothelium largely as a response to physical conditions.

SUMMARY AND CONCLUSIONS

The present contribution attempts an experimental analysis of the origin and development of blood cells and the endothelial lining cells of the vascular system. Studies on the origin of blood and endothelium in the normal embryo are rendered peculiarly difficult on account of the important rôle that wandering mesenchyme cells play in this process as well as the perplexing mixture of cells of different origin brought about by the early established circulation. The origin of no other tissue is so confused by mechanical and physical conditions.

The first difficulty has been met by a study of living Fundulus heteroclitus embryos with the high power binocular microscope. The wandering mesenchyme cells may in this way be followed to a great extent. The disadvantages due to the intermixture of cells in the blood current have been overcome by the investigation of embryos in which a circulation of blood is prevented from taking place.

When Fundulus eggs are treated during early developmental stages with weak solutions of alcohol the resulting embryos in

many cases never establish a blood circulation. In other respects these embryos may be very nearly normal and the development and differentiation of their tissues and organs often proceeds in the usual manner though at a somewhat slower rate. The heart and chief vessels are formed and the blood cells arise and develop in a vigorous fashion. The heart pulsates rythmically but is unable to propel the body fluid since its venous end does not connect with the yolk vessels and in many cases its lumen is partially or completely oblitered by periblastic material and nuclei which seem to be sucked into the heart cavity from the surface of the yolk.

In these embryos without a circulation of the blood one is enabled to study the complete development of the different types of blood corpuscles in the particular regions in which they originate. There is no contamination of the products of a given region through the introduction of foreign cells normally carried in the blood current.

The *actual* haematopoetic value of the different organs and tissues may be determined in the experimental embryos, and clearly distinguished from the ordinary reproduction or multiplication of blood cells which in the normal embryo would reach these organs through the circulation.

The debated question as to the production of blood cells from vascular endothelial cells may be conclusively answered, at least for the species here studied.

The results and conclusions derived from these experiments may be summarized as follows:

1. The Teleost embryo is capable of living and developing in an almost normal fashion without a circulation of its blood. Red blood cells may be seen to arise and differentiate in these living embryos in two definite localities, one within the posterior body region, and the other the blood islands on the yolk-sac.

The blood cells remain confined to their places of origin, yet they attain a typical red color and may persist in an apparently functional condition on the yolk-sac for as long as sixteen or twenty days. The normal embryo becomes free swimming at from twelve to fifteen days, but these individuals without a

circulation never hatch although they may often live for more than thirty days.

All recent investigators have claimed that there are no blood islands present on the Teleostean yolk-sac. Yet the presence of such islands is readily demonstrated in living Fundulus embryos, in normal specimens as well as in those with no circulation.

2. The plasma or fluid in the embryos failing to develop a circulation begins to collect at an early time in the body cavities. The pericardium becomes hugely distended with fluid as well as the lateral coelomic spaces and the Kupffer's vesicle at the posterior end of the embryo. The great distension of the pericardium due to this fluid accumulation pushes the head end of the embryo unusually far away from the surface of the yolk. The heart is thus stretched into a long straight tube or string leading from the ventral surface of the head through the great pericardial cavity to the anterior yolk surface (compare figures 15 to 20).

No blood vessels form on the extreme anterior portion of the yolk-sac, so that the venous end of the heart is never connected with veins, and does not draw fluid into its cavity to be pumped away through the aorta. When the heart cavity does contain fluid it is unable to escape and small floating particles may often be observed rising and falling with the feeble pulsations of the heart.

3. The hearts in embryos without a circulation are lined by a definite endocardium, but the myocardium is poorly developed, sometimes consisting of only a single cell layer. Chromatophores are not present in the wall of the normal heart but in the experimental hearts these large cells ladened with pigment granules are invariably found. The cavity in many of the hearts is almost if not entirely obliterated by the presence of periblastic material and large amorphous periblast nuclei.

The conus end of such hearts leads directly to a more or less closed ventral aorta, portions of the aortic arches are seen in the sections as open spaces, and dorsal aortae are almost invariably seen as typical spaces lined by characteristic embryonic endothelium.

A point of much importance is the fact that *neither these hearts with their endothelial linings nor any portion of the aortae at any stage of development have ever been seen to contain an erythroblast or an erythrocyte.* Cells of this type are completely absent from the anterior region of the embryo.

4. Pigment cells normally occur on the Fundulus yolk-sac and arrange themselves along the vascular net so as to map out the yolk-sac circulation in a striking manner. Loeb has thought that this arrangement along the vessel walls was due to the presence of oxygen carried by the corpuscles within the vessels. In the embryos without a yolk-sac circulation the pigment cells arise but rarely become fully expanded so that the usual long branched processes are represented only by short projections, the chromatophore consequently seems much smaller than usual.

The unexpanded pigment cells, however, wander over the yolk-sac and collect in numbers around the plasma filled spaces. The yolk surface of the pericardium and the periphery of the Kupffer's vesicle are often almost covered with pigment. The hearts are during early stages full of plasma and the pigment cells form a sheath around them, while pigment cells are never present on the normal hearts during the embryonic period.

These facts would seem to indicate that the plasma rather than the erythrocytes contain the substance which attracts the chromatophores and initiates their arrangement along the normal vascular net of the yolk-sac.

5. A definite mass of cells characteristic of the Teleost embryo is located in the posterior half of the body between the notochord and the gut and extends well into the tail region. This so-called 'intermediate cell mass' is the intra-embryonic red blood cell anlage in many of the species.

The peripheral cells of the mass as claimed by Swaen and Brachet or the mesenchyme about the mass, Sobotta, forms a vascular endothelium which encloses the central early erythroblasts.

In individuals without a circulation the erythroblasts arise in a normal manner in this centrally located position, and be-

come erythrocytes filled with haemoglobin. Typical vascular endothelium completely surrounds the erythrocytes which instead of being swept away as usual by the circulating current remain in their place of origin. All of the early blood forming cells of this intermediate mass give rise only to erythroblasts.

6. Contrary to the opinion of most recent observers on blood development in Teleosts, the Fundulus embryos both with and without a circulation possess blood islands on the posterior and ventral portions of the yolk-sac. These blood islands are formed by wandering mesenchymal cells which migrate out from the posterior region of the embryo. They represent all that remains of the peripheral yolk-sac mesoderm in the Teleosts and probably wander way from mesoderm related to that of the intermediate cell mass. The intermediate cell mass may possibly represent the bulk of the peripheral mesoderm which is here included within the embryonic body, while in other meroblastic eggs it is spread out over the yolk. The only mesodermal portion of the yolk-sac in Fundulus is made up of the disconnected wandering mesenchyme cells some of which group themselves to form the blood islands, while others give rise to the yolk vessel endothelium, and still other wandering cells develop into the chromatophores.

7. The non-circulating red-blood corpuscles within the embryo remain in a fully developed condition for eight or ten days and then undergo degeneration. In an old embryo of sixteen days it is sometimes found that very few of the corpuscles in the intermediate mass are still present and these are degenerate. The vascular endothelium has been lost and numerous mesenchymal cells have wandered in and lie among the corpuscles.

On the yolk-sac the corpuscles no doubt have a better oxygen supply and here they maintain their color longer but finally also present a degenerate appearance with small densely staining nuclei and cell bodies much reduced in size.

8. Vascular endothelium arises in loco in many parts of the embryonic body in which blood cell anlagen are not present. This endothelium is in all cases utterly incapable of giving rise to any type of blood cell. This incapacity cannot be attributed to the abnormal condition of the embryo as true blood cell anlagen in the same specimen produce blood corpuscles in abundance.

Vascular endothelium in the fish embryo has no haematopoetic function.

9. Neither lymphocytes nor leucocytes have been found to arise in the yolk-sac blood islands nor within the intermediate cell mass.

The embryonic white blood cells are most abundant in the anterior body and head regions, and these cells occupy extravascular positions usually lying among the mesenchymal cells.

The sources of origin of the white and red blood corpuscles in Fundulus embryos are distinct, and these two different types of cells cannot be considered to have a monophyletic origin except in so far as both arise from mesenchymal cells.

The adult blood of Fundulus contains lymphocytes and several varieties of granular leucocytes.

10. There is evidence to indicate that definite environmental conditions are necessary for blood cell proliferation or multiplication. Blood cells do not normally divide when completely enclosed by vascular endothelium. This is the key to the shifting series of so-called haematopoetic organs found during embryonic development.

Erythroblasts lying about spaces unenclosed by vascular endothelium proliferate steadily and give off their products into the spaces from which they find their way into the embryonic vessels. Should such an erythroblast be carried by the circulation to another unlined space it may become arrested there and again undergo a series of divisions giving rise to other erythroblasts. When, however, these spaces become lined by endothelium the blood cell reproduction stops.

It most embryos the earliest blood cell formation occurs in the yolk-sac blood islands. The cells in these islands continue to divide until they become surrounded by endothelium, then the yolk-sac blood islands lose their haematopoetic function and become a vascular net through which the blood circulates. The liver now takes up the rôle of harboring dividing blood cells within its tissue spaces, when these spaces become vascularized by endothelium, here again the blood cells no longer multiply but merely circulate.

Finally, in the mammalian embryo, one organ after another ceases to offer the necessary harbor for dividing blood cells until the red bone marrow is the only tissue presenting the proper relationship of spaces and vessels, and here alone the erythropoetic function exists to supply the red blood cells for the entire body circulation. The red blood corpuscles are always produced so as to be delivered into the vessels and thus very soon occupy an intra-vascular position, while the white blood cells arise and remain for some time among the mesenchymal tissue cells in an extra-vascular position.

11. Lymphocytes and leucocytes along with the invertebrate amoebocytes are all generalized more or less primitive wandering cells, and are almost universally distributed throughout the metazoa.

Erythrocytes are very highly specialized cells with a peculiar oxygen carrying function due to their haemoglobin content. In contrast to the universal distribution of the leucocytes the erythrocytes are only found in the vertebrate phylum, except for a few cases existing in some of the higher invertebrate groups. Yet even in these particular cases the oxygen carrying blood cell never presents the typically uniform appearance of the vertebrate erythrocyte. The oxygen carrying function in invertebrates is usually confined to the liquid plasma.

Typical vascular endothelium is widely distributed in the animal kingdom and appears to be formed from a simple slightly modified mesenchymal cell.

These three very different types of cells all seem to arise from mesoderm—the mesenchyme. Yet the present investigation would indicate that each arises from a distinctly different mesenchymal anlage.

The erythrocyte anlage is localized and perfectly consistent in the quality of its production.

The lymphocyte and leucocyte anlage is more diffusely arranged and not definitely localized in any particular cell group.

The vascular endothelium appears to be formed in loco in almost all parts of the embryonic body, and its formation is absolutely independent of a circulating fluid or the presence of blood cells.

The facts presented seem to indicate that vascular endothelium, erythrocytes and leucocytes although all arise from mesenchyme are really polyphyletic in origin: that is, each has a different mesenchymal anlage. To make the meaning absolutely clear, I consider the origin of the liver and pancreas cells a parallel case both arise from endoderm but each is formed by a distinctly different endodermal anlage, and if one of these two anlagen is destroyed, the other is powerless to replace its product.

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PART 2

CONTENTS

INTRODUCTION

The aim of the present consideration is an analysis of the histogenetic changes passed through by the mesenchymal cells in the living yolk-sac. A study of the origin and development of the blood and vascular endothelium in normal teleost embryos, and in other specimens in which the circulation of the blood had been experimentally prevented, made it evident that a detailed

[1] This second part of the Memoir is a continuation and expansion of the foregoing study on "The origin of blood and vascular endothelium in embryos without a circulation of the blood and in the normal embryo." The first part is referred to in the following pages as the 'previous paper.'

investigation of the development of the living yolk-sac would be most instructive for a comprehensive knowledge of the behavior of mesenchyme in forming the blood cells and vessels. The yolk-sac had been thoroughly investigated in sections and the appearance and location of the earliest blood islands and vascular formations were already familiar.

The egg of the Teleost is particularly adapted to the investigation of such a problem, since its yolk-sac has no definite mesenchymal layer and the freely wandering mesenchyme cells may be clearly seen between the ectoderm and yolk periblast. The remarkable extent to which the cells migrate and the great numbers of such wandering cells impress one with the importance of this cellular movement in embryonic development. The appreciation of this phenomenon also emphasizes the great danger of interpreting developmental processes from a mere study of serial sections. Sections fail to produce a correct impression of what is actually taking place in an area vasculosa. The study of living embryos is absolutely necessary and through it one quickly becomes acquainted with the remarkable rôle played by wandering cells in the formation of the heart and vessels, as well as the production of the future blood cells.

These facts have been pointed out long ago, but with little effect, as is indicated by the enormous literature containing the endless interpretations and guesses of numerous authors after studies of fixed and sectioned material. It is not intended to under rate the importance of the study of sections. However, such a study to be fully comprehended must be accompanied by observations on the living material as far as is practical. These observations are further greatly clarified by an experimental modification of the normal developmental processes where such is possible.

Almost thirty years ago Wenckebach ('86), at that time a young medical student in Holland, described his observations on the living embryos of the developing bony-fish. In this contribution he lamented the fact that the knowledge of the embryology of the bony-fish, as well as of other vertebrates, was based almost entirely on studies of sections of the embryos. The

germ layers were described actually as growing layers and from this layer formation the different organs were built or produced by foldings. Each cell was thought of as being passive and only through its division was the formation of the organs brought about. This initial investigation, and the only one so far as I know, by young Wenckebach gave him an entirely different view point regarding the processes of embryonic development.

Wenckebach readily observed the cells of the mesoblast independently wandering in amoeboid fashion, often with extraordinarily long protoplasmic processes, within the body of the embryo as well as upon the hypoblast-free yolk. The wandering cells move as with aim and purpose to form certain definite organs. In the formation of the anlage and further development of the heart, as well as the vessels and other structures, this independent wandering of the mesoblast cells performs a most important part.

Unfortunately, Wenckebach's scientific efforts ceased and his early study was unappreciated since on only one occasion was it considered by an investigator, Raffaele ('92), who studied similar material. Today the student gathers from text- and hand-books as well as descriptive embryological contributions much the same orthodox conception of the layers and foldings as all important factors in the origin and development of embryonic organs. No doubt the growth of layers and folds does contribute its part, but this part is almost negligible in a study of the development of the vascular system and blood.

Wenckebach and Raffaele, in their observations of the wandering cells on the yolk-sac failed to recognize the erythroblast. They were also unable to follow the processes in the differentiation of pigment and endothelial cells in the way at present possible with improved microscopes. The experimental embryos without a circulation of the blood are also most instructive for comparison in such a research.

The wandering mesenchymal cells on the yolk-sac differentiate into four distinct types of cells. The chromatophores of two varieties, one black and one of a reddish brown color, endothelial lining cells of the yolk vessels and islands of erythroblasts may

all be seen to form in the living specimens. The association of the four types is also clearly determined. In addition to these cells the large periblast nuclei are conspicuously seen. The periblast never gives rise to any type of tissue or cells, but finally the nuclei become swollen and distorted and degenerate in the manner Wenckebach long ago described.

MATERIAL AND METHODS OF STUDY

The material used for these observations and experiments was the embryo of the Teleost, Fundulus heteroclitus. The eggs of this fish are very transparent and may be readily observed by transmitted light with a high power microscope. A compound binocular microscope made by E. Leitz has been found to serve splendidly for such observations since with this instrument an oil immersion lens may be used to study the embryo suspended in a hanging drop from a thin cover glass over a hollow slide. A double or triple lens condenser facilitates the regulation of light and in a darkened field the almost transparent cells may be seen while granular or pigmented cells are distinctly outlined.

The mesenchyme cells are of sufficient size to be readily followed with a Zeiss DD objective while the eggs are grouped on the bottom of a watch glass. An ordinary microscope serves almost equally well for these observations but the Leitz binocular has the advantage of producing an apparently stereoscopic effect, since it permits the observer to look with both eyes at the same time. One is also enabled to see much better and look much more continuously than with one eye. An ordinary binocular microscope is unfit for the finer observations on account of the poor arrangements for condensing the light, and the magnification is insufficient for details of structure.

Observations have been made on the normal embryo at all developmental stages. Specimens in which the circulation of the blood was never established were also used, since in these the behavior and development of the cells of the yolk-sac are in no way contaminated by the introduction of additional cells brought in the circulating current. Such specimens enable one to hold the yolk-sac in its original condition so far as cellular elements

go, and further give an opportunity to test the influence of the circulation on the mode of differentiation and function of the mesenchymal cells.

The prevention of the circulation has been accomplished in the same manner as employed in the previous investigation and fully described in the September number of this journal. The eggs shortly after being fertilized are placed in solutions of alcohol in sea-water. The series of solutions most advantageously used is prepared as follows: 1.5 cc., 2 cc., 2.2 cc., 2.4 cc., 2.6.cc., 2.8 cc., and 3 cc. of 95 per cent alcohol is added to 50 cc. of sea-water. These solutions are renewed after twenty-four hours and after another twenty-four hours the eggs are placed in pure sea-water.

Such a series gives, of course, gradiations of the effect. Eggs in the weaker solutions develop normally in many cases, while other individuals develop slightly slower than the normal and have the circulation of their blood arrested to different extents. Many individuals in all of the solutions fail entirely to establish a blood circulation and although the heart pulsates feebly it does not propel the plasma for one or another reason which has been previously discussed. In spite of the failure of the blood to circulate, the development of the cells on the yolk-sac progresses in an almost normal fashion and vessels and blood corpuscles arise in this region and may be carefully observed throughout the life of the embryo.

The observations on the living yolk-sac have been supplemented by a study of fixed and cleared specimens. Embryos at different stages of development are fixed in a saturated solution of corrosive sublimate to which 5 per cent of glacial acetic has been added. Eggs are left in this mixture for 4 or 5 minutes, then rinsed in tap water and placed in 10 per cent formalin. The formalin is changed after about one-half hour when it has become slightly cloudy. This method if carefully handled brings out in a most beautiful way the cell outlines of the ectoderm of the yolk-sac (fig. 34). The yolk remains rather transparent and the mesenchymal cells may be observed beneath the clear cut net-work formed by the ectodermal cell borders. This method has been frequently employed by other workers and I have used

it myself for ten years on Fundulus eggs, but have never before succeeded in getting this heavy outline of the cells. It seems scarcely possible that so striking an appearance could have been overlooked, yet it is perfectly simple to obtain. Fundulus yolk-sacs fixed in this way are equally as beautiful as silver preparations of cell boundaries.

In addition to the above, I have used for the first time another solution which renders the specimen still more transparent. This is a mixture of strong formalin 5 parts, glacial acetic 4 parts, glycerine 6 parts, and distilled water 85 parts. Eggs are placed directly into this and left for two days, and then transferred to 10 per cent formalin for permanent preservation. The fluid mixture causes the egg to swell to some extent but it leaves the yolk as clear as in life and by fixing the cells causes them to stand out in beautiful contrast (figs. 5, 6, etc.). The mixture of glycerine and glacial acetic has been used for a long time in preparing transparent specimens of invertebrate eggs. Wilson in 1892 used it with Nereis eggs. The proportions here employed have been used by several students at Woods Hole and are not original with me, except that others leave the eggs permanently in the mixture while it seems better to put them in formalin after two days. The eggs remain equally transparent in formalin.

The cleared specimens are most valuable for use in connection with the studies of the living. But the remarkably beautiful filamentous processes of the wandering mesenchyme cells and the endothelial lining cells of early vessels are not so extensive as during life. Some shrinkage or contraction of these processes always accompanies fixation. The movement of the processes in life also gives one a much better conception of their form and structure.

THE EARLY WANDERING CELLS

About two hours after fertilization the eggs of Fundulus heteroclitus have undergone the first division and are in the two-cell stage. The cleavages then continue in a more or less regular fashion to form a discoidal mass of cells as a cap on the yolk. At eighteen to twenty hours the germinal disc is begin-

ning to flatten or thin out in order to begin its expansion to cover the yolk sphere. After the fourth or fifth cleavage some of the peripheral cells of the germ disc are somewhat fused with the yolk mass and do not present a clearly formed distal cell wall. The nuclei of such cells continue to divide and begin to wander or are pushed out into the superficial yolk material. In this way are formed the so-called periblast nuclei, or more correctly periblast syncytium, of the teleost. This periblast syncytium precedes the germ disc in its descent over the yolk, so that one observes loosely scattered nuclei of unusually large size forming an advance border around the periphery of the germinal disc. The nuclei multiply and finally lie scattered over the entire yolk surface by the time the germ ring or blastodisc has completely covered the yolk (figs. 5, 7 and 8).

These periblast nuclei are of interest to us in the present consideration only on account of the fact that they are located in a superficial syncytium covering the yolk. It is over this syncytium that the mesenchymal cells wander. The periblast of the hypoblast-free yolk-sac of the teleost, in so far as position is concerned, may be compared to the endodermal covering of the yolk-sac in other meroblastic eggs.

The outer cover of the yolk-sac in Fundulus is formed by the germinal disc as it grows over the yolk. This constitutes the ectoderm of the sac which is its only true or typical layer. Thus the yolk-sac consists of an outer-continuous ectodermal layer beneath which are freely wandering mesenchymal cells and below these the periblastic syncytium fuses into the yolk material itself.

The periblast nuclei were interpreted by Agassiz to represent the survival of the nuclei which had at one time in phylogeny controlled the segmentation of the yolk. These were the nuclei of the former yolk laden cells in the holoblastic cleavage of the ancestral teleost. Others have thought that they played some part in the formation of the ventral wall of the gut, etc. In Fundulus, however, they take no part in the formation of the body tissues or organs, but may be observed to degenerate in the late embryo. The periblast nuclei become very much vacuolated,

irregular in shape and huge in size before their final degeneration. After the embryo has hatched the remains of the yolk contain a protoplasmic mass in which the periblast nuclei are packed together and the whole is finally absorbed.

The blastodisc is separated from the yolk by a space which arises during the early hours of development. This space between the ectoderm and periblast has been interpreted by Agassiz and Whitman ('84), Ryder ('87), Wilson ('90) and others to represent the blastocoel or segmentation cavity. It is actually into this space that the wandering mesenchymal cells migrate and we shall later recall this fact as of importance in interpreting the nature of the vascular lumen in connection with other body cavities or spaces.

Twenty-four hours after fertilization the germinal disc is from one-quarter to one-third way over the yolk-sphere. Its wall has thinned out centrally and around the periphery is seen a thickened border, the so-called germinal ring. After about 48 hours the germ-ring has traveled almost completely over the yolk-sphere and now surrounds the small remaining uncovered pole of the yolk which may be considered a yolk-plug. A shield-shaped thickening, beginning about the twenty-fourth hour, extends from one region of the germ-ring towards the animal pole. This is the embryonic shield and along its median line a second thickening begins to appear which is the first indication of the embryonic body.

From the edges of the embryonic shield and from the germ-ring as it finally encloses the yolk, an early migration of mesodermal cells takes place. The cells apparently do not wander far and some of them may again be included in the embryonic body. After the germ-ring has enclosed the yolk, from 45 to 50 hours usually, a very active migration of mesenchymal cells begins from the caudal and posterior lateral portions of the embryo. Figure 1 shows outlines of a few such cells wandering out from the side of an embryo of 45 hours. This figure is a camera sketch from life and even at this early time there are some cells inclined to be more or less spindle or stellate in shape with long delicate filamentous projections, while other cells are

of more irregular shape with short amoeboid processes. Figures 3 and 4 show the cells highly magnified and in active movement; figure 3 the spindle-shaped delicate process type and figure 4 the heavier more amoeboid cell. These cells are actively wandering and changing in shape as figure 4 shows. From *A* to *E* are the outlines presented by a single cell at five minute intervals.

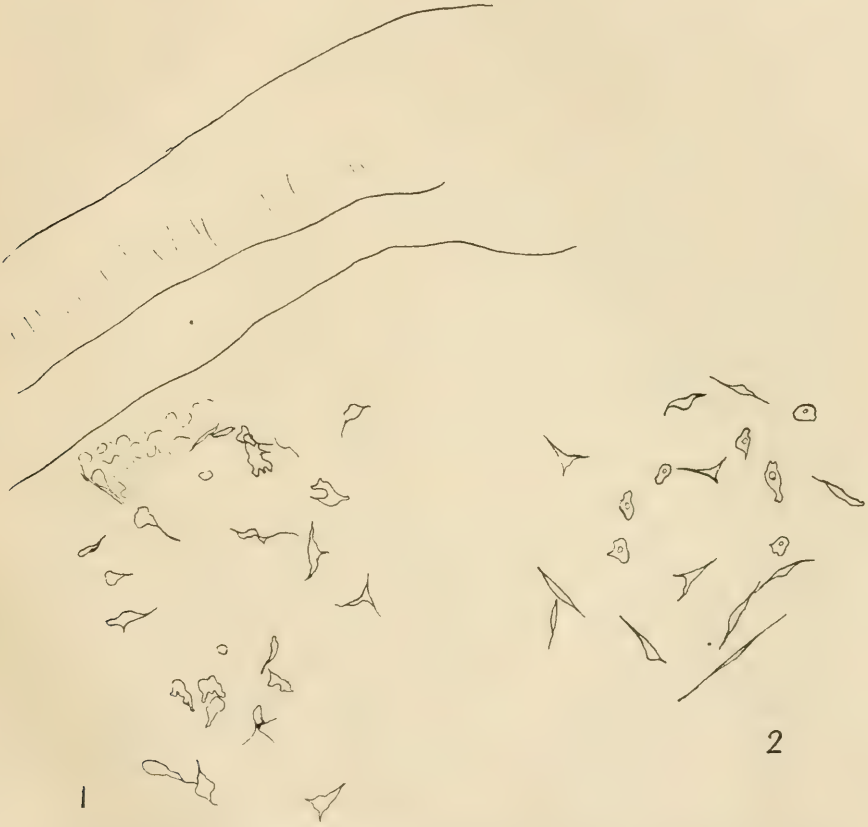
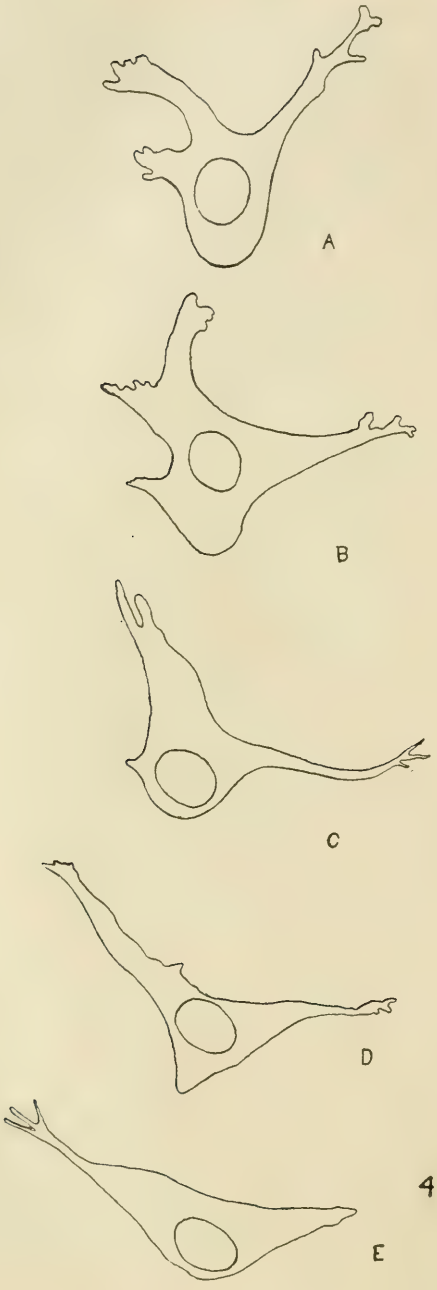


Fig. 1 A group of mesenchyme cells indicated in outline, camera lucida sketch, wandering out from the side of an early embryo, 45 hours old. Two kinds of cells are seen, one with delicate filamentous processes and another amoeba-like cell.

Fig. 2 A similar group in one microscopic field from an embryo 48 hours old, again showing the two types. The elongate spindle cells are future endothelial cells.



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It is, therefore, difficult to state that the spindle-shaped cells may not change to the heavier amoeboid pattern and vice versa. But we shall see that these two forms are the probable if not actual forerunners of two groups of cells later, at any rate, with permanent shapes and structures differing in much the same way as the early appearances now differ. Figure 2 again shows a sketch from life of wandering cells on the yolk-sac of a 48 hour embryo, and here as usual the spindle cells are contrasted with the amoeboid ones. The spindle cells are assumed to be future vascular endothelial cells and the amoeboid cells are probably the future chromatophores of the yolk-sac.

The places from which cells wander out most actively are the borders of the tail, and particularly from that mass of cells representing the obliterated germ-ring. Figure 5 shows the cellular arrangement around the tail end of a 48 hour embryo fixed and cleared. The cells have withdrawn their processes. The open space in the cell group at the tip of the tail, *yk*, is the place still remaining between the borders of the germ-ring. Later, the tail of the embryo grows over this cell group so that it is less conspicuous. Figure 8, the tail end of a 56 hour embryo, shows such a condition.

The important fact which we shall later consider is that these cells form a mass continuous with the mesenchymal cells within the tail end of the embryo. The wandering cells may be interpreted to grow out from the end-bud or blastopore lip. They are a scant rudiment of the peripheral or ventral mesenchyme usually growing away from the blastopore lip over the yolk mass in the reptile and the bird. It will be presently shown that such an interpretation is upheld by the nature of the products to which these wandering cells give rise.

Figure 6 illustrates the wandering away of cells from the lateral mesoblast of an embryo with two pairs of somites, 48 hours old. Figure 7 shows the head end of a 56 hour embryo. Scarcely any

Fig. 3 Camera outlines of wandering mesenchyme cells 48 hours old, all of the future endothelial type, highly magnified. *A* and *B* are two outlines of the same cell at a 6 minute interval.

Fig. 4 Camera outlines of one cell drawn at 5 minute intervals *A* to *E*. The cell is a migrating future chromatophore in an embryo 50 hours old (3b. DD ob.)

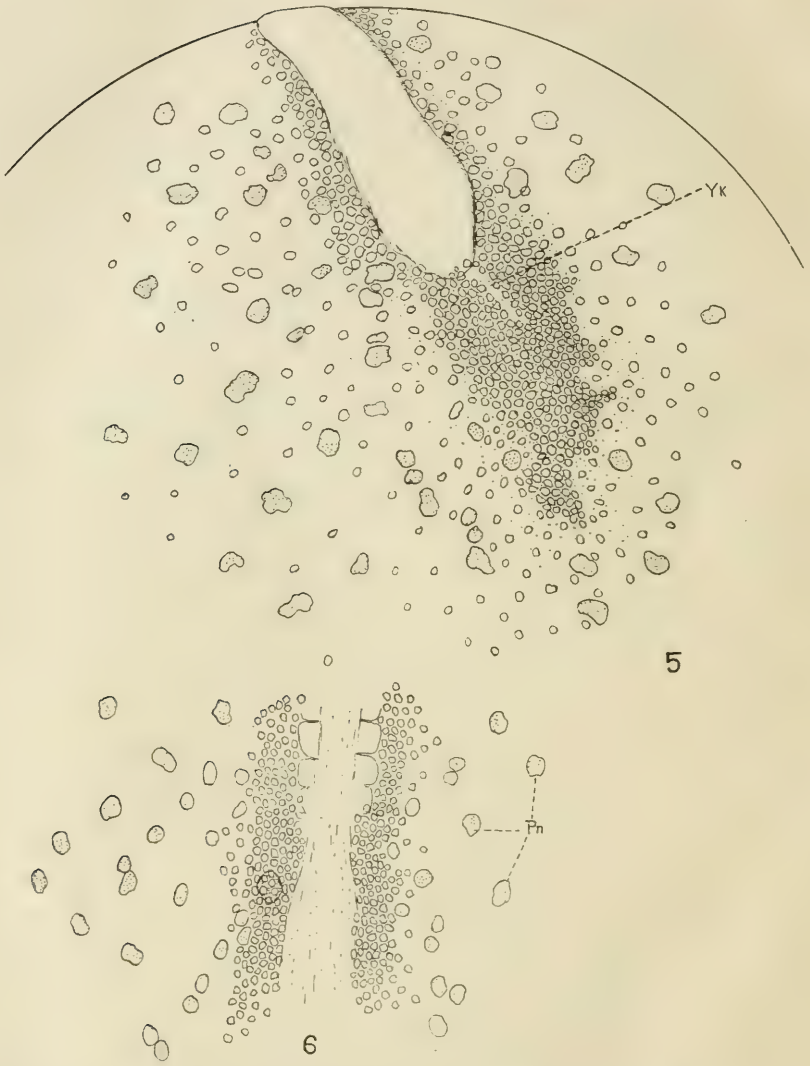


Fig. 5 Camera lucida outline of the tail end and caudal yolk region of an embryo 48 hours old, fixed and cleared in glycerine-formalin. The germ ring just closing over the yolk pole, numerous mesenchyme cells beginning to wander away from the caudal end, huge periblast nuclei indicated in outline and stipple over yolk. *Yk*, polar bit of yolk just being covered by union of germ-ring border.

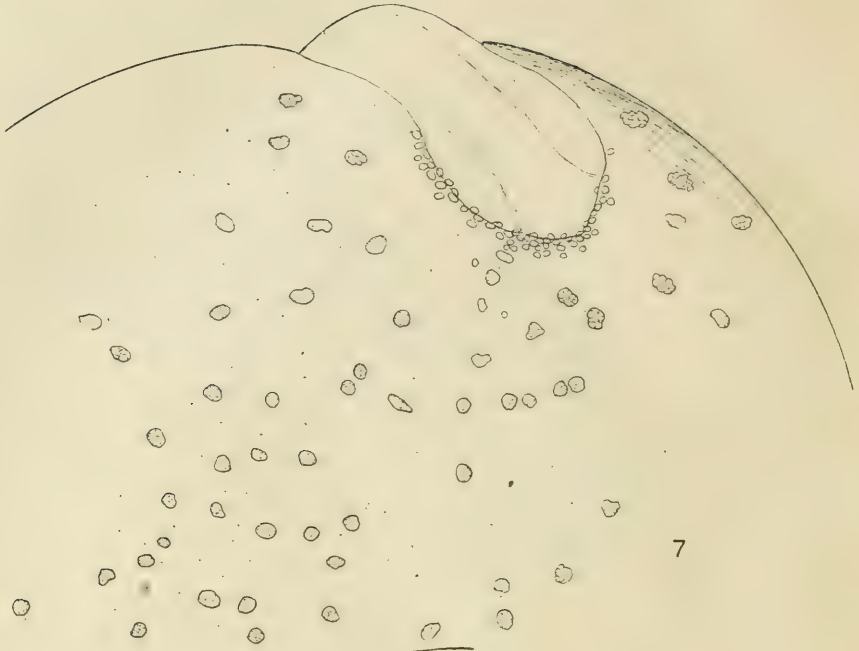
Fig. 6 Portion of the caudal half of an embryo of 48 hours showing the first two pairs of somites recently formed, cells of the lateral plate mesoderm extend out upon the yolk as shown in outline, some beginning to wander away. Periblast nuclei outlined and stippled. Glycerine-formalin specimen.

cell migration is taking place from this region. A few mesenchyme cells are found along the border of the head; these cells later take part in either the formation of the heart or pericardial wall. The tail end of the same embryo, figure 8, shows a remarkable contrast; here there is an enormous wandering out of cells from the mesoblast of the embryo. The two figures show the huge periblast nuclei to be widely distributed throughout the surface of the yolk sphere. These drawings are from cleared specimens and the cell outlines are more or less circular without the beautiful processes characteristic of the living.

The tail end of a living embryo 72 hours old, some time before the blood began to circulate, is illustrated by figure 9. The circle beneath the tail represents Kupffer's vesicle. The various shaped mesenchymal cells are represented in the act of wandering out over the nearby surface of the yolk. The embryo and yolk are beautifully transparent in life and the cells are clearly seen as they move upon the surface of the periblast.

An entire embryo, except the anterior portion of the head which extends beyond the curve of the yolk, is shown in figure 10 at a lower magnification. This specimen was 76 hours old when drawn. The heart had begun to contract slowly and feebly but no circulation of fluid had begun. Groups of mesenchymal cells are seen wandering away from the lateral and particularly the caudal regions of the embryo and are now scattered broadly over the yolk surface; there being very few, however, in the anterior region. The lateral plates of the mesoderm are seen at the sides of the head, and a circle at the caudal end indicates the Kupffer's vesicle which is always clearly shown at this stage.

In embryos of 72 hours, and somewhat earlier, there are wandering out from the tail region a number of cells slightly smaller than the two types mentioned above. These small cells tend to be more or less circular in outline but show slow amoeboid movement as they send out short blunt processes. They group themselves into small clumps and are to give rise to erythroblasts or future red blood corpuscles in the yolk-sac as shall be discussed beyond. Figure 31, page 569, shows six such cells from the living yolk-sac of an embryo 90 hours old:



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a circulation was partially established in this specimen but these cells had not yet been taken into the vessels.

The various wandering cells then represent the mesodermal layer of the yolk-sac in the teleost. They never assume a membranous layer-like arrangement, but finally differentiate into the characteristic structures of the yolk-sac. As is shown by the illustrations, these cells are very numerous and during their earlier stages are actively changing their shape and moving over the yolk surface.

We may now consider the further development of such cells the living embryos.

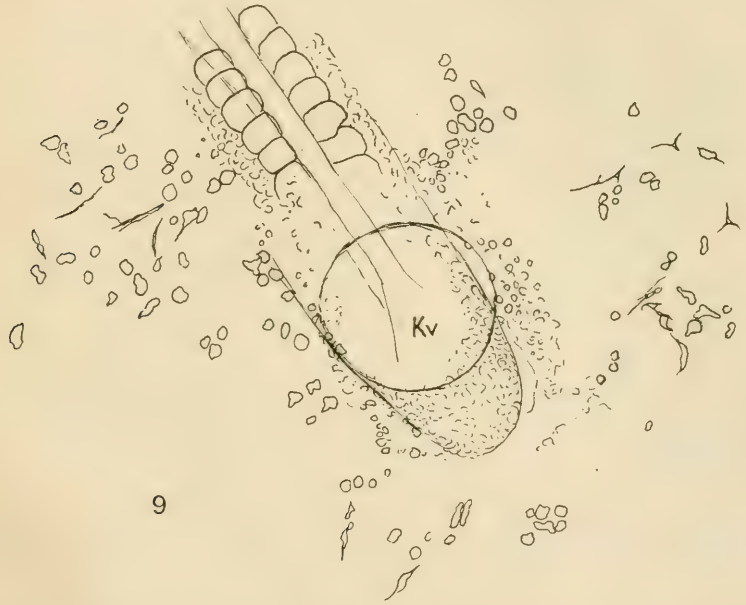


Fig. 7 Outline of the head end of a 56 hour embryo, scarcely any wandering mesenchymal cells in this region. Large periblast nuclei scattered over yolk surface.

Fig. 8 The caudal end of the same embryo; note the great contrast in the abundance of out-wandering mesenchymal cells. Glycerine-formalin specimen.

Fig. 9 The caudal end of a living normal embryo of 72 hours, with beautifully delicate mesenchymal cells wandering away from the body; *Kv.*, Kupffer's vesicle (3b. 2/3 ob.).

Fig. 10 A camera sketch of an entire embryo of 76 hours except the anterior end of the head. The mesenchymal cells wandering away from the tail region and to a less degree from the sides of the body.

DEVELOPMENT AND DIFFERENTIATION OF THE WANDERING CELLS

1. *Chromatophores*

a. The black type of chromatophore. The first to be considered of the four types of cells which develop on the yolk-sac are the black chromatophores. These are the largest and most conspicuous cells of the yolk-sac. In the early stages discussed above, one notes even in embryos of 2 days that certain cells of the yolk mesenchyme are considerably larger than others.

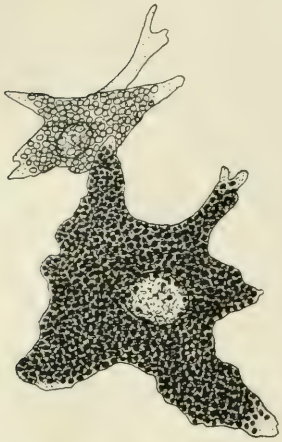
These large cells may be followed through their development and they will be found to differentiate into one or the other of the two types of chromatophores. The amoeboid cell shown in different stages of movement already referred to as figure 4, from a 52 hour embryo, is of this large type, and concluding from my observations on great numbers of embryos, this is an early condition of the future black chromatophore before any pigment granules are deposited.

Slightly older stages, figures 22 and 26, show the same cells containing a light amount of pigment granules. Between the second and third days the pigment granules appear and in an embryo 72 hours old, end of the third day, the chromatophores are already well differentiated freely moving huge cells.

A black chromatophore from an embryo 72 hours old is shown in figure 11 with one of its processes overlying the body of a brown chromatophore, the type to be considered in the following section. The black cell is loaded with coarse granules. The nucleus occupies a central position and is clearly shown on account of the displacement of the pigment granules by its transparent body. Several pseudopod-like processes project from the chromatophore which is actively moving. The clear cytoplasmic tip of the pseudopod extends beyond the granular mass.

Figures 12 and 13 are two other illustrations of the same cell after 15 minute intervals. Its shape is constantly changing and it is slowly moving in a direction towards the right side of the page. The brown pigment cell is also moving and their rates of progress are indicated by the increasing distance between them.

This movement of the chromatophores continues until about the end of the fourth or middle of the fifth day in the normal embryos. By this time all of the black pigment cells of the yolk-sac with few exceptions have taken up more or less permanent positions along the walls of the blood vessels or around the surface of the pericardial space. The individual chromatophores have increased enormously in size as is seen by comparing figures 11, 12 and 13 with figure 14, all drawn at the same magnification, though figure 14 is one-third more reduced in reproduction.



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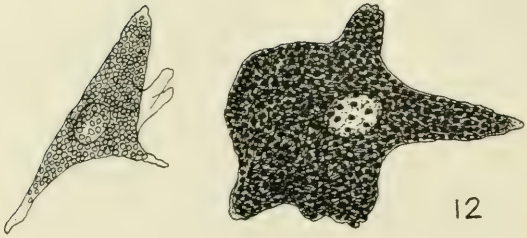

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It must be appreciated, however, that some of the difference in extent is due to the flattening of the cells in figure 14.

Figure 14 shows two huge pigment cells on the yolk-sac of a 5 day embryo in the act of arranging themselves along a vessel wall. The granules are not so densely arranged as in the younger stages, since the cell body is greatly thinned out in pressing around the vessel. A number of granules are often arranged in solid black lines and masses as indicated in the figure.

The two cells are close together and a very peculiar phenomenon is taking place. Each cell sends out short processes to meet similar processes from its neighbor. The processes fuse, and finally the two cell bodies melt into one thus forming a pigmented syncytium about the vessels of the yolk-sac. The syncytia continue to expand along the vessels as enclosing sheaths (fig. 15). The dense black of the young chromatophores becomes a steel grey as the granules are more thinly spread along the vessels.

In order to test whether the cells had actually joined or fused to form a true syncytium, I attempted to contract them, thinking that this should pull them apart unless they were actually united. The various solutions of KCl which Dr. Spaeth has found to contract the chromatophores within the embryo's body failed entirely to produce any change in the chromatophores of the yolk-sac. Solutions of adrenalin of one to 1000, one to 10,000 and one to 100,000, which Dr. Spaeth so kindly supplied me, were then tried. These solutions contract the pigment cells on the brain of the embryo until they appear as small black dots, but neither the black nor brown chromatophores on the yolk-sac respond in the slightest degree. Such specimens were preserved to show the extreme contraction of the chromatophores over the brain of the embryo in contrast to the unchanged pigment cells of the yolk-sac.

Fig. 11 A black and brown chromatophore lying in contact on a yolk-sac of 72 hours. The black cell is much the larger with broader pseudopod-like processes; both are in active movement as shown by comparing figure 12, of the same two cells 15 minutes later and figure 13, the same cells 20 minutes after figure 12. (3b. DD. ob.).

From this it would seem as though the material of the chromatophore had lost its contractile or wandering power after once becoming arranged around the yolk vessels. Those black chromatophores which retain their cellular individuality along the borders of the pericardial space also fail to contract when treated with KCl or adrenalin.

Although this physiological test failed to serve the purpose for which it was used I feel certain, after many observations, that the black chromatophores actually do form true syncytial masses as they surround the vessels.

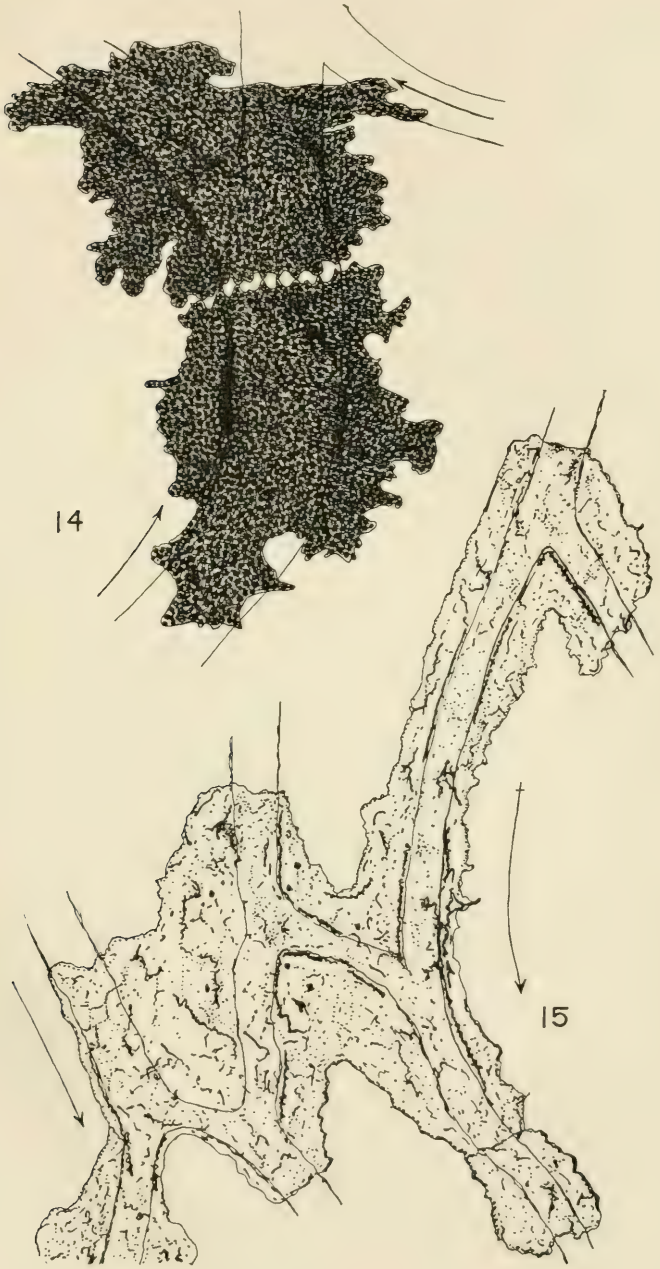
b. The brown type chromatophore. The brown chromatophores differentiate on the yolk-sac at about the same time as the black. They are always somewhat smaller and more delicately formed cells than the black, and react in a slightly different manner. Figures 22 and 26 show several brown chromatophores before the end of the third day. They are paler in appearance and more elongate in shape than the black cells.

The two types of cells are well contrasted in figures 11, 12 and 13 referred to above. The brown cell is smaller, with more delicate processes and is the more rapidly moving of the two. The three figures indicate its condition in embryos of 72 hours.

These pigment cells also wander to the vessel walls and yolk spaces and take on their permanent condition about the fifth day. Figure 16 illustrates one of the exquisite brown pigment cells in a yolk-sac of 5 days. The nucleus is still distinguishable in life while it is not in the black cells of this age. The mossy branched processes projecting from all sides give to this cell a most fascinating form.

Fig. 14 A camera lucida drawing of two huge black chromatophores lying upon a yolk vessel of a 5 day embryo. The adjacent sides of the chromatophores are beginning to fuse to form a syncytium. The direction of blood flow is indicated by the arrows.

Fig. 15 A syncytial mass of black chromatophores forming a sheath about the vitelline vessels. The chromatophores become so thin that the pigment granules are spread apart giving a less intense color. The individual cells are completely lost in the syncytium (3b. DD. ob.).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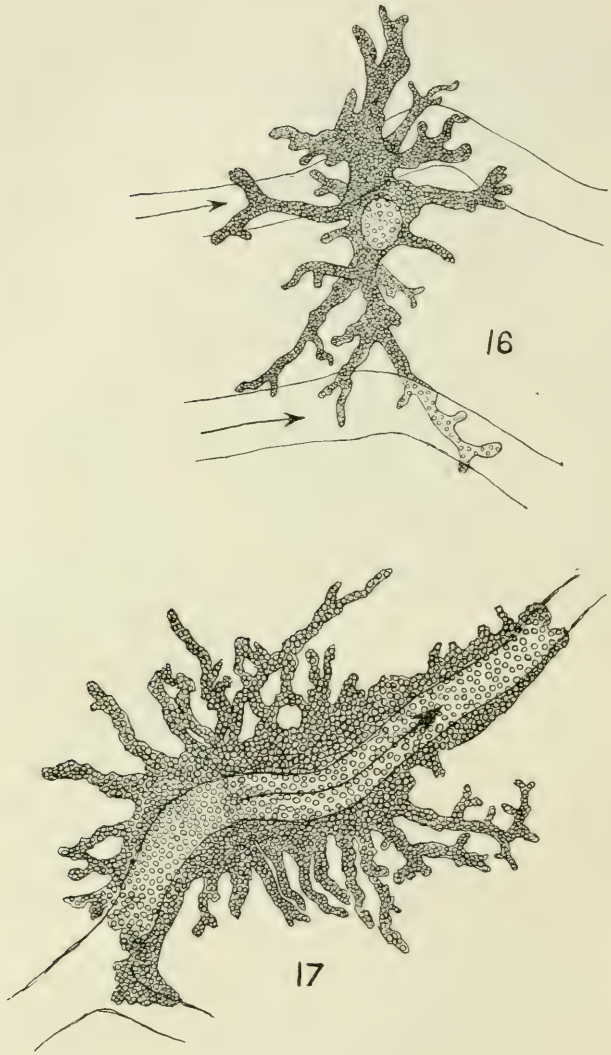


Fig. 16 A brown chromatophore on the yolk of a 5 day embryo. The cell is coming in contact with two vessels shown in outline. The moss-like processes extend from all sides of the cell.

Fig. 17 A similar cell 12 days old surrounding a yolk vessel. The complex processes from this cell are quite in contrast to the almost smooth border of the black chromatophores of figures 14 and 15. The brown cells never fuse to form syncytia.

Finally, in older embryos the cell body often surrounds a vessel, as shown in figure 17, but the processes persist and project from it in all directions, forming a striking contrast to the more or less smooth outlines finally assumed by the black cells, figures 14 and 15, as they surround the vessels.

The brown chromatophores do not group themselves together or form a syncytial mass as the black pigment cells are prone to do. They remain individually separated and many really never become associated with vessel walls, but lie scattered on the yolk surface.

In early embryos, from 72 to 90 hours, the brown pigment cells may sometimes, though rarely, get into the blood stream. I have never been able to observe one in the act of entering the current. Yet in a quiescent state they might become surrounded by endothelial cells along with the erythroblasts, and finally be swept away. They might, on the other hand, actually migrate through the porous wall of an early vessel.

The enormous brown pigment cell presents a smooth circular outline as it is carried along in the blood current. On account of its size the chromatophore often meets with difficulties in passing narrow portions of the vascular system. Several such cells were seen in the blood circulation of different embryos during the course of the observations, and when once located in the current the same cell could be seen periodically for a long time as it came around again and again through the vesssel within the field of study. There is no question of the identity of these cells, as their characteristic reddish brown color and coarse granular structure is readily recognized. It is most improbable to think of them as becoming changed into any type of blood corpuscle, and it is doubtless entirely by accident that they occasionally become entrapped within the vessel wall and washed away by the current.

c. Behavior of the chromatophores in specimens with no circulation. The behavior of both the black and brown types of pigmented cell is distinctly different in embryos without a circulation of the blood from that described in the two previous sections for normal embryos.

During the early stages, up to the beginning of the fourth day, the cells wander in amoeboid fashion much the same as in ordinary specimens. In other words, at this time the condition is the same in all embryos since the blood has not begun to circulate in any. At about 72 hours the blood circulation begins in the normal embryos and the pigment cells seem to be attracted to the vessel walls, as already pointed out. If the circulation does not begin at this age, the plasma accumulates in various spaces, chiefly the pericardial sac and Kupffer's vesicle at the caudal end of the embryo. The excessive accumulation of plasma in these spaces causes them to be in many cases hugely distended. The heart in such specimens also becomes a sacular structure filled with plasma which it is unable to pump on account of one or another deficiency in the vascular system.

Large numbers of chromatophores of both types tend to aggregate about these plasma filled spaces and partially cover their walls. The spaces are thus rendered more conspicuous. In some specimens this coating of the distended plasma sacs by pigment cells is most remarkable, but such an arrangement is not invariable and in a number of individuals the pigment cells are irregularly scattered over the yolk-sac with no recognizable pattern or system.

The heart of embryos in which there is no blood circulation is almost without exception covered with chromatophores. These cells often form a perfect sheath about such hearts whether the heart is a plasma filled sac or a mere string. The patterns of these arrangements are illustrated by numerous figures, particularly figures 15 to 20 in the previous paper.

A point of much interest in this connection is the fact that the heart of the normal embryo is entirely free of pigment cells.

The behavior of the chromatophores of the yolk-sac in normal individuals where they tend so decidedly to arrange themselves along the blood vessel walls along with their affinity for the plasma filled spaces in the non-circulating condition would seem to indicate that the chromatophore was attracted by the plasma itself, or some element which it contains. The distended plasma filled heart in the non-circulating cases is covered with pigment

as would be expected, yet the solid string-like heart present in many such specimens is also covered with pigment though it of course is entirely empty of plasma. In this last case, however, the string-like heart actually stretches as an axis through the pericardial space which is distended with fluid. The cells arrange themselves around the wall of the pericardium and on reaching the venous end of the heart migrate along it and so cover the heart string or tube in their effort to come in close proximity to the plasma. The distended condition of the pericardium may in this way account for the pigment arrangement along the heart in the cases with experimentally arrested circulation.

The normal heart is constantly pumping the plasma through itself, yet pigment cells are never present in its wall since they are all arranged along the vessels of the yolk-sac. In non-circulating cases many vessels form on the yolk-sac and some become quite well developed, while others actually surround the blood corpuscles of the yolk islands. Such vessels are at times covered with pigment but probably through accident as the pigment is irregularly scattered over the entire yolk-sac. Yet the pigment cells on such vessels never arrange themselves in the definite sheath-like fashion characteristic of the vessel pigment in the normal embryo.

Figure 18 illustrates the lack of arrangement of the chromatophores on the yolk-sac of a 16 day embryo without a circulation; compare figures 15 and 17 of pigment in the normal embryo. Figure 35, see beyond, also illustrates in a striking way the irregular grouping of black chromatophores in the neighborhood of a collection of stagnant blood islands in an embryo of 14 days that never had a circulation of its blood.

All of these reactions cause one to wonder what is the actual function of the pigment cells upon the yolk-sac. The entire egg is rather transparent and their function might be to protect the vessels from the light, yet the vessels are never completely covered and the development of the eggs in the dark is normal but not in any way supernormal.

The pigment cells form such a complete sheath about the vessels in some cases that one might be led to imagine that their

expansion and contraction would serve as vaso dilator and contractor. Yet when they are along the vessel wall I have failed to see them contract or expand even when treated with substances such as KCl and adrenalin that violently contract the chromatophores within the embryonic body. These experiments have not been carried sufficiently far since they were directed towards another point, still they indicate at least that the pigment sheath of the vessel wall does not respond as a delicate vaso-motor apparatus.

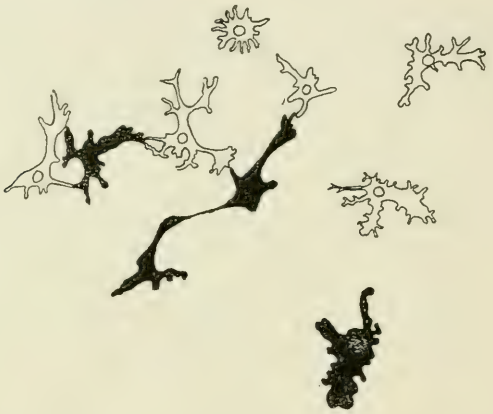


Fig. 18 A group of brown, indicated in outline only, and black chromatophores on the yolk-sac of a 16 day embryo in which the blood has never circulated. There is no arrangement of the pigment cells on vessels and no real syncytium of black chromatophores as compared with the conditions in the normal embryo.

Wenckebach ('86) found in certain pelagic eggs containing a number of oil drops which invariably floated up that pigment cells often completely surrounded the oil globules, and as he thought prevented these globules from focussing heat or light on parts of the embryonic body. The oil drops in the demersal Fundulus yolk do not particularly attract the pigment cells and they are rarely found to lie against the oil globule.

The function of the pigment in the yolk-sac of Fundulus, if it has any function other than its own existence, is most difficult to determine. The same is true of the abundant pigment in the coelomic wall and other internal structures of many animals.

d. Relationship of chromatophores to blood and endothelial cells. There has been much discussion in the literature regarding the relationship of the chromatophores to blood corpuscles and to endothelial cells. The actual relationship of these cells is clearly brought out by a careful study of the living yolk-sac in Fundulus. The cells are completely different and their structures when once established are consistent in their particular type.

The black chromatophores, the brown chromatophores, the endothelial cells and blood corpuslces are all derived from mesenchymal cells which wander away mainly from the caudal region of the embryo during early stages of development. These cells come to lie in the primary segmentation cavity of the yolk-sac beneath the ectoderm and over the periblast syncytium. The mesenchymal cells very soon begin to show certain differential characters in structure and behavior. When certain ones of the cells incline in a definite direction their development progresses continuously along this line.

Observations on the normal living embryos and comparison with those individuals without a yolk-sac circulation lead one to conclude as follows regarding the wandering mesenchymal cells. At the time these cells leave the embryo proper to wander over the yolk, differentiation has proceeded to some extent in the embryo and the mesenchyme cells are probably somewhat limited in their potentialities. Certain of them are derived from the same portion of mesenchyme that gives rise to the intermediate cell mass or future red blood cell forming mass within the embryo. This mass is located towards the caudal end of the embryo and the wandering cells derived from it finally come to lie on the posterior and ventral surfaces of the yolk-sac and form islands of red blood corpuscles. Few if any of these cells reach the anterior regions of the yolk-sac before the circulation of the blood begins. In embryos that *never* have a circulation the blood islands lie beneath the tail of the embryo and on the ventral yolk surface. The future endothelial cells wander out from the caudal end and side of the embryonic body and finally line up to form vessels in a manner to be described beyond. The pigment cells also wander out from the lateral regions and differentiate into chromatophores of either the black or brown variety.

It would seem that these cells must have some potential differences at the time they come to lie in the yolk-sac, since from that time on they all appear to be in an identical environment. Two cells lying side by side in the yolk-sac above the periblast and beneath the ectoderm would be expected to develop and grow in similar fashion unless there were some internal difference between them. I have thus concluded that the mesenchymal cells which wander in the yolk-sac of the Fundulus embryo must be potentially of four different classes when they first wander out, although all have the ordinary appearance of embryonic mesenchymal cells. Otherwise, it is difficult to conceive why they should develop into four distinct types of cells while all are surrounded by an identical environment so far as is possible to discover. Differentiation in various directions must be due either in the first place to similar cells developing in different chemical or physical surroundings, or in the second place it may result from potentially different cells developing under identical conditions.

The four types of cells are all derived from mesenchyme, just as the thyroid follicles and pulmonary epithelium are derived from endoderm but from different endodermal anlagen, and further than this there is no relationship. Pigment cells and blood corpuscles are perfectly separate and distinct types derived from different mesenchymal analgen and are not in any way transmutable.

2. *History of the endothelial cells*

The endothelial cells on the living yolk-sac of Fundulus embryos are readily recognized. Their entire behavior in the formation of the earliest yolk vessels may be traced in a manner to fully repay the patient observations necessary in order to follow through the processes.

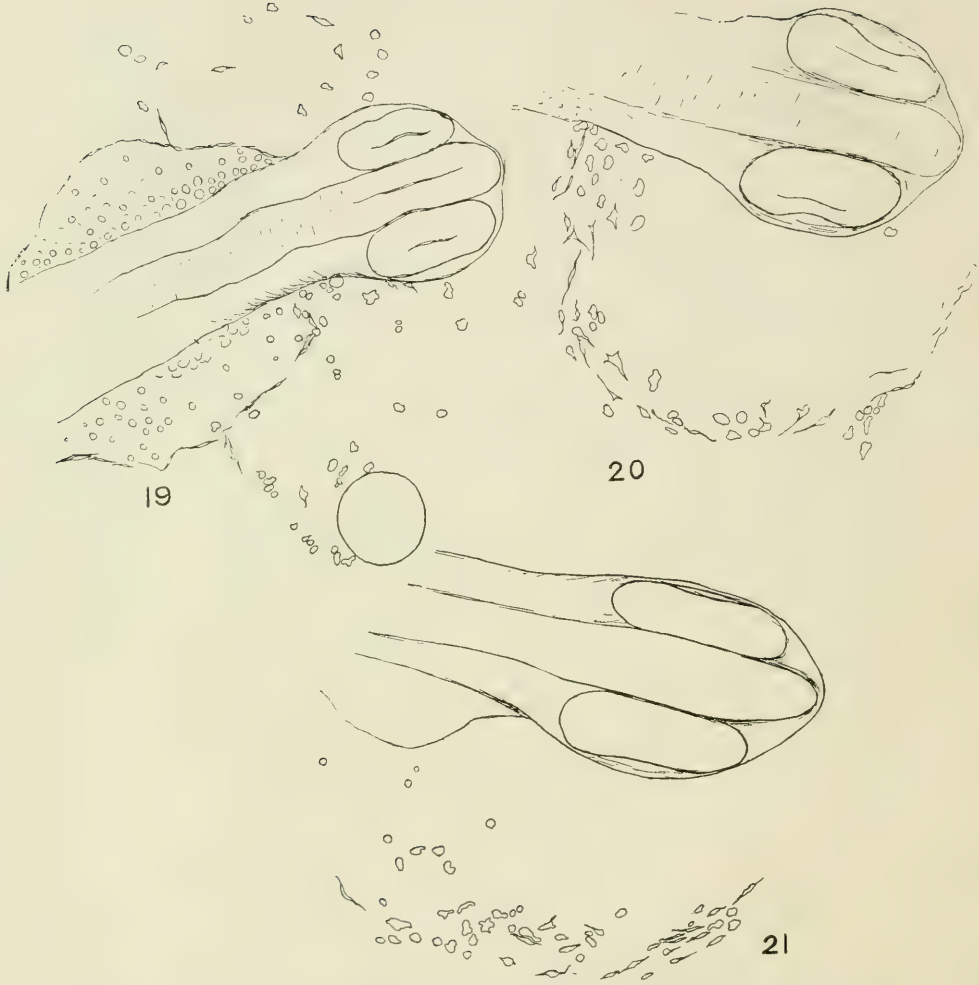
Among the early wandering cells migrating away from the lateral borders and caudal end of the embryo it is noted that certain ones assume a delicate spindle shape with filamentous processes extending from their ends and occasionally projecting

from their long sides. In a 48 hour embryo these cells already present the appearance of individual endothelial cells. They migrate indefinitely for a few hours and then tend to group themselves in more or less irregular collections.

Up to this time no one from mere observation could be absolutely certain that the cells of this rather characteristic appearance are actually to become vascular endothelial cells in all cases. The possibility, of course, exists that the elongate spindle cells may at times round up and then assume the more amoeboid shape of the probable future chromatophore. Yet since the shape of these cells is so characteristic and such shapes are so constantly present, one is inclined to believe that the same cell may actually retain this character until it really becomes a component part of a vascular endothelial arrangement.

Figures 19, 20 and 21 illustrate the region along the side of the embryo's head at 48 hours old. It is in this region that the first large yolk vessel develops. This vessel carries blood from the body of the embryo around a short circuit to reach the venous end of the heart and thus in a way relieves the flow that otherwise would force itself through the small poorly formed vessels in the embryonic body. This vessel is, therefore, of necessity one of the earliest to develop. The three figures, 19, 20 and 21, show variations in the arrangement of the wandering mesenchymal cells in the region of the future vessel.

In figure 19 there is really no definite cell aggregation except along the edges of the head mesoblast as it spreads somewhat over the yolk, yet a few of the cells show the typical spindle shape. Figure 20 indicates a tendency of the mesenchymal cells to line themselves in a group exactly along the course of the coming vessel. Many of the cells in this group give the actual appearance of an endothelial cell after it is fully developed and forming one of the units in a vessel wall. The embryo illustrated by figure 21 shows much the same condition. Very few mesenchymal cells occur between this cell aggregation and the side wall of the head. Lateral of the vessel group the cells are also not numerous and have no system of arrangement.



Figs. 19, 20 and 21 Outlines of the head regions of three living embryos from 48 to 50 hours old, showing different conditions in the grouping of mesenchymal cells on the yolk which later give rise to the large vessel that short circuits blood from the side of the embryo around over the yolk to the venous end of the heart. The future vessel wall is now separate spindle shaped mesenchyme cells.

This cellular aggregation may then be regarded as the actual anlage of the vascular endothelium of the future vessel. The anlage consists merely of a group of separate wandering mesenchyme cells, and not of a capillary net in any sense.

A slightly older embryo shows a still more definite alignment of the mesenchymal cells and still later presents the appearance of cellular strings or cords as illustrated in an embryo of 67 hours by figure 22. Here the wandering mesenchyme cells have differentiated to such an extent that they are readily distinguishable as black and red chromatophores and elongate endothelial cells.

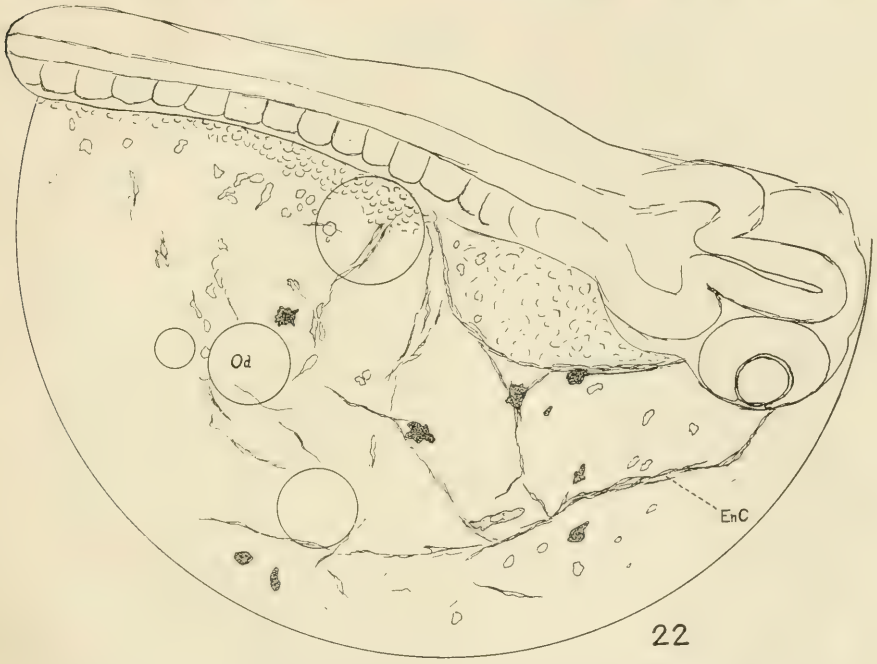


Fig. 22 A sketch of a 67 hour embryo showing the stage in the origin of yolk vessels in which the mesenchymal cells have a linear arrangement. Early black and brown chromatophores are also shown in the yolk-sac. *Od*, oil drops.

Early erythroblasts are also seen on the caudal region of the yolk-sac in such an embryo, but are not shown in the aspect here illustrated.

The endothelial cells are strung out in various directions and several linear groups are more or less isolated from the rest. The string to be the future large vitelline vessel is not clearly continuous posteriorly, but anteriorly it is well outlined extending towards the venous end of the now forming heart which has not yet

begun to pulsate. Here there is no further doubt that these elongate spindle cells are the elements which will make up the endothelial lining of the vessel wall.

There is considerable variation in the rate of development of the yolk vessels in embryos of the same number of hours. Some individuals may be in the condition just described, while others of this age may have already begun to establish a circulation of the blood. Figure 23 shows the yolk region lateral of the head in another embryo at 67 hours. In this specimen an incipient circulation has begun and the cord of cells illustrated in figure 22 has now become a small hollow tube sufficiently open to allow the passage of a single file of corpuscles from the side of the embryo around to the venous end of the heart. The individual cells composing the vessel are distinctly seen and their nature is clearly made out with a higher magnification. They retain the same general appearance presented before entering into the vascular arrangement.

Near this vessel is shown in figure 23 a partially formed vascular plexus which is broken in several places and entirely disconnected from the large vein through which the blood is flowing. There is of course no circulation of fluid in this partially formed plexus.

Figure 23 was sketched with a camera lucida at 12 M. and about three hours later at 2.45 P.M., figure 24 was sketched from the same field. The main vessel in accord with Thoma's ('93 and '96) first law of vessel growth has increased in calibre on account of the increased flow and pressure of the circulation. It now permits the passage of three or more corpuscles abreast and is a strongly developed vessel. The former disconnected vascular plexus has grown towards the large vessel and two of the projections shown in figure 23 have now met the wall of the vein and joined with it. One of the first corpuscles from the circulation to enter the plexus is shown in the figure tightly held in the small vessel. Immediately opposite this vessel a sprout is seen growing away from the wall of the large vein.

Figure 25 illustrates the state of arrangement at 6.00 P.M., three hours older than figure 24. The third process from the

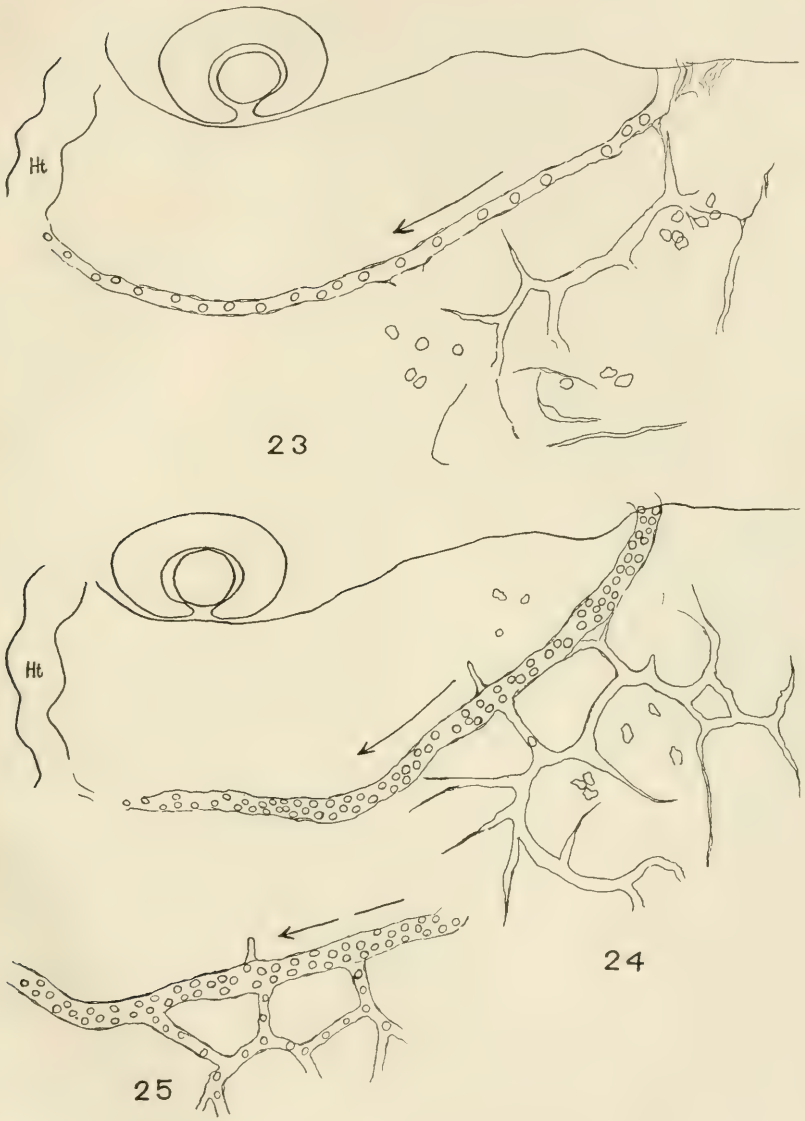


Fig. 23 The large vitelline vein in an embryo of 67 hours just beginning to permit the passage of blood through its lumen. Corpuscles moving in single file. This becomes the largest vessel of the embryo and arose from the arrangement of the freely wandering mesenchymal cells of figures 19, 20 and 21. In the

plexus has here joined the vein and corpuscles are freely passing into the vessels. The sprout from the vein wall is still seen opposite the entrance of the middle vessel. The small plexus arose in loco entirely independently and subsequently became connected with the larger vessel which also arose as we have seen from a group of mesenchymal cells.

Figure 26 shows another lateral view of the head region of an embryo of 67 hours in which the large vein is filled with circulating corpuscles and the beginning of the same plexus followed in figures 23, 24 and 25 is seen lateral of the vein. At this stage the plexus is entirely disconnected and separated from the vein.

In the formation of the large yolk-sac vein, as well as all other vessels arising upon the yolk, there is nothing to be seen of the forerunning capillary plexus so strongly emphasized by Thoma in the yolk-sac of the chick. There is no selection and dilation of certain channels in the capillary plexus of the teleost's yolk-sac to form the veins. Here the veins seem to arise in rather definite localities and soon expand into their full form after the circulation has become established.

This method of the formation of vessels was beautifully brought out by Wenckebach ('86) in the early study already referred to so often. He concludes: "Aus diesen Beobachtungen geht hervor, dass Mesoblastzellen durch selbständige amoeboïde Bewegungen die Wände der Blutgefässe des Dotters bilden." Raffaele ('92) later confirmed this observation and further was strongly of the opinion that in selachians and other vertebrates a similar process of vessel building from wandering cells also takes place.

From my present studies on the normal and abnormal Fundulus embryos, I can see no way to doubt that the endothelial wall of the primary yolk vessels in the bony-fish is formed by arrangements of wandering mesenchymal cells.

lower part of the camera sketch is shown an independent capillary plexus not yet connected with the vein. *Ht*, heart.

Fig. 24 The same vessels 2 hours and 40 minutes later. The main vessel has increased in caliber and two branches of the capillary net have joined the vessel.

Fig. 25 The same vessel three hours later, a sprout is given off opposite the union with the middle capillary and corpuscles now enter all three capillaries. The arrows indicate the direction of blood flow.

Wenckebach's description cannot be fully agreed with in all detail. He thinks, for instance, that cells forming part of the vessel wall are brought by the blood stream. These cells have small protoplasmic processes but they are not in any way to be confounded with the "definitiven Blutkörperchen." Such cells have never been observed in the Fundulus embryos and if they exist, which is very improbable, their part in vascular formation is extremely insignificant.

Wenckebach observed the three primary vessels on the yolk to bud and give off sprouts forming other vessels. The wandering cells also formed small separate tubes which later became connected to form a portion of the vascular net. By these methods the complex vascular net of the yolk-sac was finally formed. This agrees closely with what may actually be seen to occur in the embryos of Fundulus.

Considerable variation occurs in the position of vessels and a number of actual abnormalities are found in which the bilateral arrangement is completely disturbed. These abnormalities are frequently very instructive for a thorough understanding of the origin and development of the yolk-vessels. Occasionally, a group of cells will form a completely isolated endothelial space which may fail to associate itself with a vessel. Figure 27 shows such an isolated space in a yolk-sac of 90 hours old; solid endothelial tips project from the space, yet it is completely isolated so far as can be determined with the highest power, and at the same time every part of it is clearly and distinctly seen.

When the early yolk vessels are studied under high magnification, the individual cells may be clearly observed and they are strikingly the same as before they became associated to form the vessel. The cells are not closely arranged but distinct intervals and spaces exist between them and filamentous processes often project far into the lumen and may actually at times fuse with a similar process from a cell on the opposite side of the wall. These filaments thus stretch across the vessel and may even persist after the blood has begun to flow. They are well seen by focussing so as to get an optical section through the cavity. (Or-

Fig. 26 Outline of the yolk vascular condition in the head region of a 67 hour embryo. Blood is circulating freely in the large vessel but the yolk net of vessels is not yet connected with the current. Early black and brown chromatophores are also shown.

Fig. 27 An isolated endothelial cavity with solid projecting tips, no connection can be seen with any other vascular spaces. From an embryo of 90 hours.

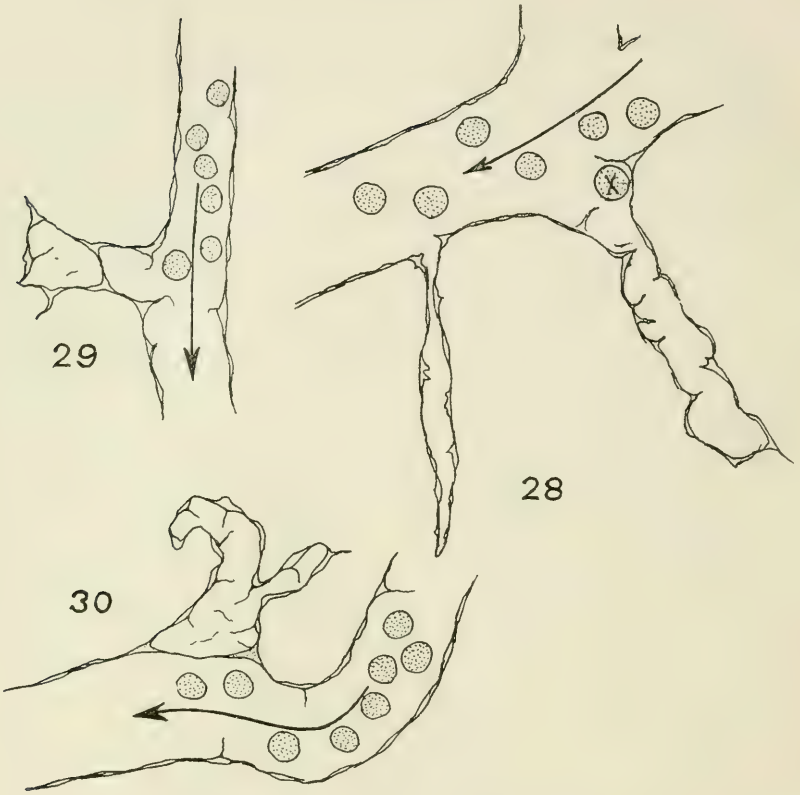
puscles often strike against the cell processes and cause them to wave back and forth as the current flows past.

The cells of the vessel wall thus maintain much of their individuality and may actually separate themselves or loosen away from the small growing tip of a vessel. The tips of the vascular sprouts probably break up or disassociate in this manner to include small groups of corpuscles which may be seen to enter the vessels from the yolk surface.

A most instructive specimen for a study of the cellular elements of the vascular endothelium is one in which the circulation has just begun. The vessels in such an embryo are still growing in length and sprouting off branches rather actively. Figure 28 illustrates such a vessel with its incipient branches. Corpuscles are passing through the vessel in the plasma current; one of these, X, is seen harbored behind an endothelial cell at the base of the outgrowth to the right. This corpuscle remained in position for more than one hour, being protected from the current by the projecting endothelial process. Such a condition is frequently seen and conveys some idea of the actual irregularity of the vascular wall.

The cells constituting the walls of the outgrowths from the vessel are changeable in shape and doubtless move their positions to some extent. The cells at the tip of the growing sprout may be seen to send out processes as if they were actually creeping along. The behavior of these vessel walls is strikingly similar to that which Clark ('09 and '12) has so clearly described for the growing lymphatics in the tail of the tadpole.

Figure 29 shows a similar vessel with a projecting bud. The cells of this bud are seen to exhibit a most indefinite arrangement; their processes project across the space and join the cells of the opposite side and none are completely elongated or flattened as are the cells of the main vessel wall. The tip cells might still be described as stellate mesenchymal cells. The appearance shown in figure 30 is much the same. The walls of these early vascular buds are extremely loose membranous arrangements with irregularities in their surfaces and openings and spaces between the cellular components.



Figs. 28, 29 and 30. Portions of vessels from three yolk-sacs of 6 day embryos. The vessels show blind endothelial sacs projecting from their walls. The constituent cells of these sacs are distinctly seen, and still retain their wandering mesenchymal characters. Filamentous processes from these cells may extend entirely across the lumen and fuse with processes from the cells on the opposite side of the wall. Corpuscles are often entangled in the filaments as well as the spaces between the endothelial cells. *X*, a resting corpuscle harbored behind an endothelial projection.

These porous or incomplete endothelial walls permit the blood cells to occasionally escape from the vessel cavity and become free within the space of the yolk-sac; or, on the other hand, a growing vascular tip may be observed at certain stages to come in contact with a group of erythroblasts, or actually a blood island unsurrounded by vascular endothelium. The tip of the

vessel seems to disorganize to some extent and its cellular elements slowly surround the group of corpuscles which are later taken into the circulation as the current becomes established in the including vessel.

Unfortunately, I have never been able to observe the consecutive steps in any one case of this kind, so that an absolutely positive statement cannot be made at present. Yet numerous observations of the contact of vascular sprouts with groups of uncovered corpuscles and the ends of such sprouts containing corpuscles, as well as other arrangements, would indicate that the behavior of the endothelium in surrounding the naked groups of erythroblasts on the yolk-sac probably takes place about in the manner just outlined.

Figure 32, page 568, illustrates a highly magnified field on the yolk-sac of a 90 hour embryo. This field shows a very interesting composite of the vascular condition at such an age. The rapidly flowing blood current is freely passing through the vessel on the right. A short circuit is forming across below the curve of an arch in the vessel. This small vessel permits only a single line of corpuscles to pass. At this time only one corpuscle has entered and it is caught in the narrow tube. This corpuscle remained fixed for a long time and so enabled a comparison of its structure and appearance with the erythroblasts forming the group just below the huge black chromatophore. The cells of this group are uncovered by endothelium. On the left of the figure a portion of a vascular net not yet connected with the circulating current presents the typical appearance of an early blood vessel formation. The individual cells are loosely associated and the tip projecting towards the right slowly changes position. This tip later approached the group of erythroblasts and finally these cells were all included within the vessel by a process which, as stated above, I was not able to follow definitely.

After closely studying these early vessels in a large number of living yolk-sacs, the observer is able to establish very clearly the actual relationship between the vascular endothelial cells and the erythroblast or early blood corpuscles. The corpuscles on the yolk are always of a distinctly different shape and size, and

lie, as described below, in small groups with originally no endothelial cells around them. The groups are later either surrounded by endothelium or taken into the early vessels as already indicated. Nothing has been observed during a long study of these cells on the living-yolk sac of normal embryos with a free circulation, or on the yolk-sacs of embryos experimentally prevented from establishing a circulation, or finally in sections of embryos of various ages, that would indicate even a tendency of endothelial cells to change into any type of blood corpuscle. The endothelial cell, whether in the free and wandering mesenchymal state or constituting a part of the vessel wall, presents a typical shape and clear appearance entirely distinct from the wandering erythroblasts.

In observing the early yolk vessels certain things may be seen which are most important in interpreting sections supposedly showing the transition of vascular endothelial cells into erythroblasts or primitive blood cells. Frequently, one or more corpuscles become entangled within the spaces and filaments existing between the cells of the vessel wall. Other corpuscles flowing in the current strike these entangled ones and beat against them sometimes for hours before they become disentangled from the vascular pits and holes, to flow again in the current. This is an extremely common sight during the early hours of the circulation of blood in any of the yolk vessels.

It may now readily be imagined that if the embryo was killed and fixed while the corpuscles were tangled in the spaces of the vascular endothelium, a study of sections might produce the impression that the cells of the vessel wall were "protruding into the lumen and assuming the typical characters of primitive blood cells," according to the description of many that imagine the occurrence of such things. I have previously offered another explanation of these appearances and many phenomena observed on the living yolk-sac bear out the point of view. It may sometimes happen when the vascular endothelium encloses a group of primitive corpuscles that one or more of these future blood corpuscles come to lie in the plane of the vessel wall, or may actually seem to form one of the cellular components of the wall.

When the circulation begins, however, this cell becomes loosened away from the wall for mechanical reasons, the lack of long processes, etc., and projects into the lumen to be finally washed away. Any one may readily observe such occurrences who will study the living yolk-sac of Fundulus with a high power microscope and a strong condenser so as to use a darkened field.

All of these observations lead one to conclude that the only connection between vascular endothelium and primitive blood cells is one of association. The endothelial cells never metamorphose into blood cells. It is important here to recall the fact previously emphasized by the author that in those specimens in which there is never a circulation of the blood or plasma the vascular endothelium develops in a perfectly normal manner in the aorta and other intra-embryonic vessels, as well as in the vessels on the yolk-sac, yet in none of these does one find any appearance indicating a tendency of the lining cells of the vessel wall to change into any type of blood cell. Numerous other details from my notes might be enumerated which would bear on this question, but sufficient care has been taken to definitely establish the above crucial points as facts. This may not of course hold for all types of animals but it does for those studied.

I have seen a number of sections on which other investigators have based their claim that vascular endothelial cells do change into primitive blood cells and although inclined towards the acceptance of such a view from a mere acquaintance with the literature, a study of such material has convinced me that the negative interpretation is equally plausible in all cases.

3. *Blood corpuscles on the yolk-sac of teleost embryos*

In all meroblastic eggs except those of the teleost a great sheet of mesoblast is found extending over the yolk as the so-called peripheral or ventral mesoderm, or subvitelline mesoderm. It is this peripheral mesoderm that gives rise to the blood islands of the yolk-sac in selachians, reptiles, birds and mammals The teleost, however, presents a unique case in that the ventral mesoderm does not spread out over the yolk but is included

within the embryonic body. The differentiation of this mesoderm within the embryo is much the same as that of the peripheral ventral mesoderm in the yolk-sac of other groups. Thus in the bony-fish the great bulk of blood formation takes place within the so-called intermediate cell mass, the probable homologue of a portion of the yolk-sac mesoderm of other vertebrates.

The intermediate cell mass first described by Oellacher ('73) and later fully studied by Ziegler ('87), Swaen and Brachet ('99, '01), and numerous others, is derived from the primary lateral plate mesoderm, separating away from the median border of this plate. The cell masses of the two sides remain apart in some species and form the future cardinal veins and red blood corpuscles, while in others the two masses unite in the median line to form the conjoined cardinals or stem vein, which is loaded with the primitive erythroblasts—the red blood anlage.

All recent workers on the development of the blood in the bony-fish have considered this intra-embryonic blood formation as being the only source of blood cells in these animals. Several authors, however, Swaen and Brachet among them, have recognized that the cells of the stem vein may become so packed and crowded within the embryo that masses of them are directly pushed out laterally upon the yolk. They have also thought it possible that a very few cells might wander upon the yolk-sac and there form blood, but this has been considered questionable in all cases. No one has recognized the actual occurrence of blood islands upon the teleostean yolk-sac. Even Wenckebach in his study of living embryos, although he made so many important observations on the formation of the periblast and yolk vessels, entirely overlooked the early or primitive yolk-sac blood cells. This is probably due to the fact that he studied only normal embryos. In embryos without a circulation of the blood one observes the yolk islands much more readily as the cells finally become filled with haemoglobin and present a bright red color. When they are once located in these experimental embryos it becomes much easier to trace them in the younger normal individuals, and finally the observer readily locates these cells and may follow completely their migrations and association to form the yolk-sac blood islands.

The arrangement of these wandering cells in the yolk-sac, figures 7, 8 and 10, suggests in a way the regions of growth of the yolk-sac mesoderm in other meroblastic eggs. About the caudal region there is an 'area opaca' formed by the great number of wandering cells, while around the head end the scarcity of mesenchymal cells might be considered an area pallucida.

All of the yolk-sac blood islands in the Fundulus embryos are formed from certain of the early wandering mesenchymal cells on the yolk. During the early wandering stages when the future endothelial cells have a spindle shape and the future chromatophores are large amoeboid cells, other mesenchymal cells on the yolk are small and more or less circular in outline. These small circular cells move slower than the other types and throw out short thick pseudopod-like processes.

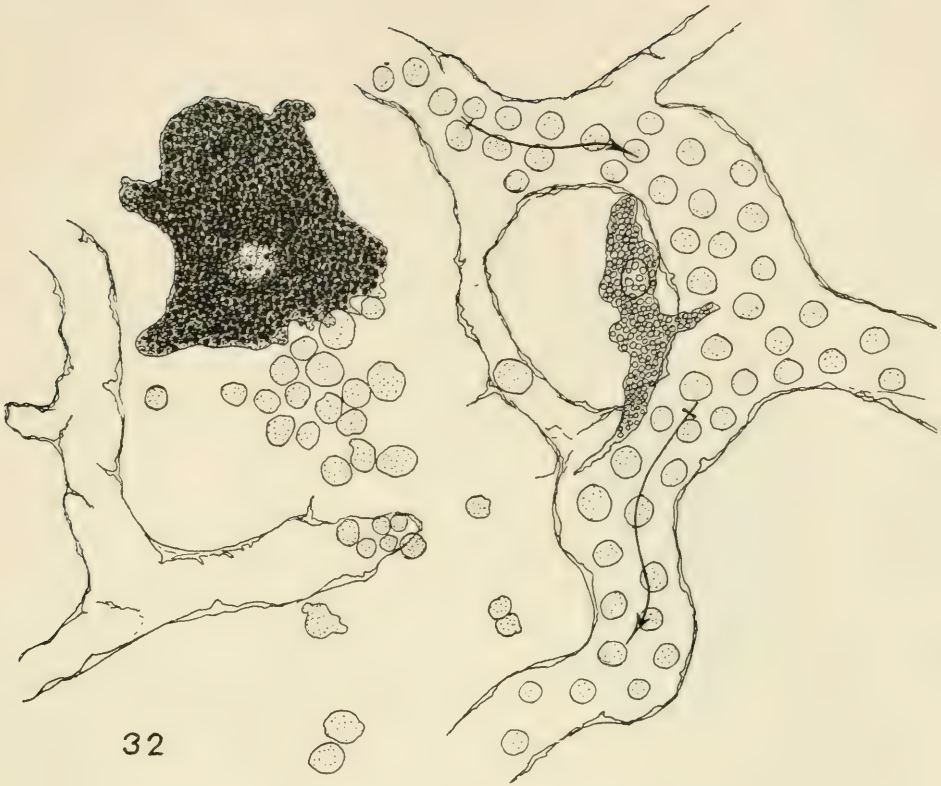
Whereas the spindle shape cells wander away from the embryo along its entire lateral border as well as the caudal end, and the large amoeboid future chromatophores have almost an equally extensive place of origin, the small round cells wander out only from a limited region. The earliest ones of these to be seen are near the caudal end of the embryo before the tail fold has completely separated the caudal end from the yolk surface. As the tail is moulded free from the yolk-sac, the point of outwandering of the circular cells follows the place of union between the ventral wall of the tail and the yolk-sac. Just at this place the mesenchyme of the embryonic body extends itself out on to the yolk as free wandering cells.

In a study of sections this mesenchyme is found to lead directly to the end-bud, Endknospe, which may be considered to represent the blastopore lip. The cord of mesoblast which has been designated as intermediate cell mass leads caudally to the end-bud which is well out in the tail. The ventral cells from this mass wander away into the yolk-sac from the extreme caudal position and other cells also wander away laterally from the intermediate cell mass. Figure 8, the tail end of an embryo 56 hours old, illustrates very well the place of outwandering of the round cells. Few, if any such cells wander out from the lateral borders of the embryo in regions more cephalad than this.

This confined local origin of the round cells and the period at which they wander out, along with their general appearance, lead one to believe that these cells are actually derived from the same general mass or group of cells which goes to form the intermediate cell mass or red blood anlage within the embryo. All of these slowly wandering circular cells finally differentiate into red blood corpuscles, as described below, just as cells of the intermediate cell mass will finally do.

In this connection a most instructive defect is frequently found among embryos developing in the stronger solutions of alcohol. Many such embryos are of the common short type with their tails split, cauda-bifida, figure 4 of the previous paper. This defect is due to the fact that the germ-ring descends over the yolk in a slow or arrested fashion and may never succeed in fully enclosing it. The caudal end of the embryo is thus divided and the two tail moieties remain spread apart laterally along the line of the germ-ring. This condition renders the caudal portion incapable of including all of its usual median tissues and such cells extend past the angle of the split and lie between the two parts of the bifid tail. The interesting thing is that the cells constituting the blood-forming intermediate cell mass lie in just this position.

Figure 33, a diagram, illustrates the embryonic body with a bifid caudal end. The great lake of blood corpuscles is situated beyond the angle of the split tail. Such an abnormality liberates the future blood forming mass from the body of the embryo and the mass spreads posteriorly over the yolk surface, yet not in a diffuse manner since it maintains its densely packed cellular structure. We might consider that here the evolutionary events are reversed. The blood anlage in the primitive fishes, the selachians, is spread over the yolk in the area opaca. In the normal teleost this primary yolk-sac blood anlage has been included within the embryonic body and localized in the intermediate cell mass. While in the abnormality here considered the intermediate cell mass is again outspread upon the yolk somewhat suggestive of the old ancestral selachian arrangement. This abnormality, in other words, may give some notion of the actual



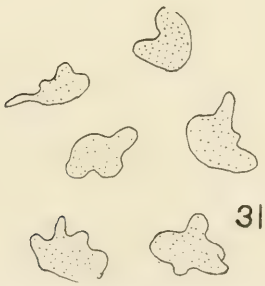


Fig. 31 A group of six early erythroblasts unsurrounded by endothelium on the yolk-sac of a 90 hour embryo. Short amoeboid processes project and the cells move very slowly.

Fig. 32 A camera lucida sketch of a microscopic field on the yolk-sac of a 90 hour embryo. All four derivatives of the wandering mesenchymal cells are shown. The circulation is established in the vessel to the right and the current follows the direction of the arrows. To the left is a small vessel not yet connected with the current; its wandering endothelial tip is approaching a group of erythroblasts still uninclosed by endothelium as they lie near the huge black chromatophore. A brown chromatophore is seen on the large vessel.

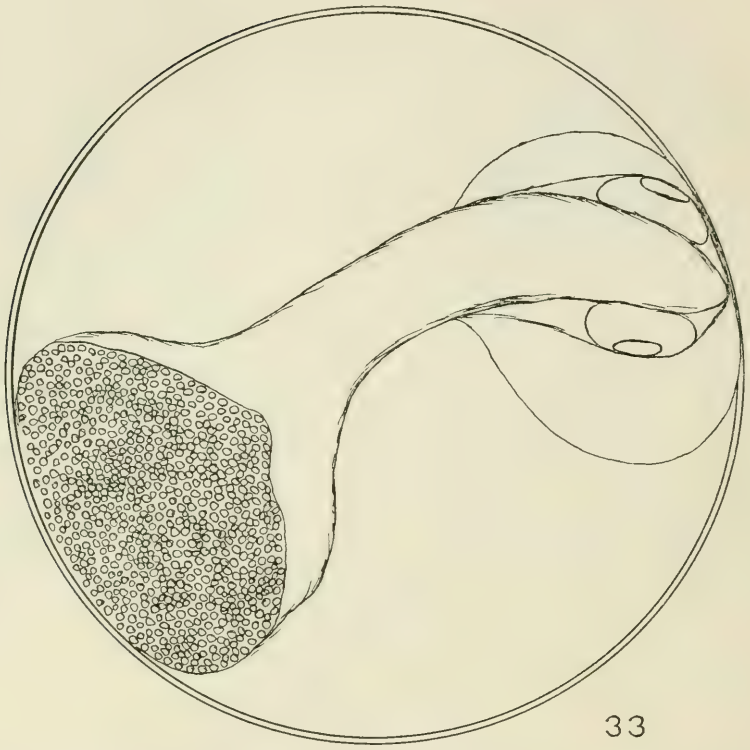


Fig. 33 An outline of a short 6 day embryo from a strong alcohol solution. The embryo presents a cauda-bifid condition and the normally intra-embryonic blood-forming mass is represented by a densely packed expanse of corpuscles outside the body of the embryo and spread upon the yolk.

incorporation of the primitive blood-forming mesoderm of the yolk-sac into the body of the teleost embryo.

The situation may be pictured as follows. In the reptiles and birds, for example, the peripheral mesoderm is outspread over the yolk and in it differentiates the blood islands of the area vasculosa. The peripheral mesoderm in Fundulus and teleosts generally does not become outspread over the yolk, but is concentrated into a median cord or mass within the caudal half of the embryo. Yet even here there is actually a tendency for the cells of this mass to be attracted to the regions of the yolk-sac, and during the early stages of development many cells sepa-

rate from the mass and wander freely on the yolk. The extent of such wandering is probably variable in different species. Yet in all eggs with an extensive vitelline circulation the wandering of these future red blood cells probably takes place to a considerable extent in spite of the fact that the cells have been so generally overlooked. A fact easily accounted for when one realizes that most of the studies in blood origin in teleosts have been confined to sections of the embryos of the salmon and trout. Sections are extremely slow in revealing the significance or even existence of wandering cells in development.

We may now consider the individual wandering cells and their subsequent differentiation. Figure 31 shows a group of six such cells. They are small when compared with the huge chromatophores but are about as large as the endothelial cells, though completely different in shape, texture and behavior. Figure 32, a camera lucida sketch, serves well to illustrate the relative sizes of the different type cells on the yolk-sac. In this figure are shown all four types, the enormous black chromatophore, the very large brown chromatophore, the delicate elongate endothelial cells of the vascular wall with their filamentous processes, and the almost circular erythroblast, small when compared with the first two types, but as large or even larger than the endothelial cell.

Figure 34 was drawn from an embryo that had been fixed in such a way as to render conspicuous the cell outlines of the ectodermal layer of the yolk-sac. Below the ectoderm groups of erythroblasts forming blood islands are shown, and the extremely small size of the erythroblasts in comparison with the enormous dimensions of the ectodermal cells is most striking.

As mentioned before, the erythroblasts tend towards a circular shape but send out short processes, while they move in a sluggish amoeboid fashion. The cytoplasm of these cells is slightly greyish and not so perfectly transparent as that of the spindle cells; this difference between the two types is not so marked during the early stages but is readily noticeable in embryos of 80 or 90 hours.

The future erythroblasts very slowly wander away from the tail region of the embryo and down the posterior surface of the

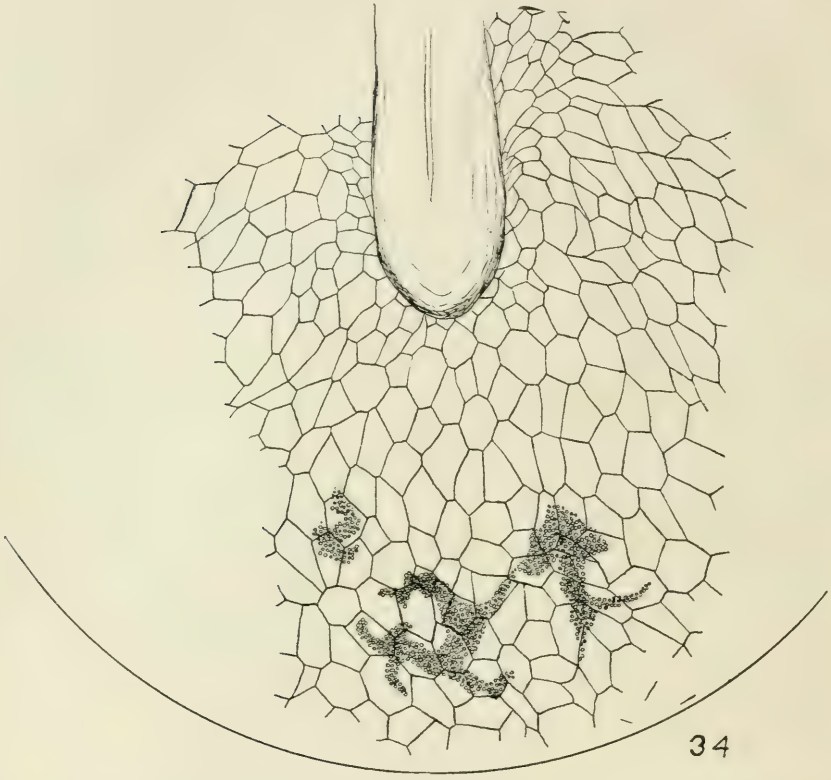


Fig. 34 A camera lucida outline of the caudal region of the yolk-sac in a 96 hour embryo with the ectodermal cell borders made visible by mercury fixation. An island of blood corpuscles is indicated by small circles which give some idea of the minute size of the erythrocytes as compared with the huge ectodermal cells.

yolk to reach its ventral surface. At various places on the posterior and ventral yolk surfaces the cells collect into groups and become less active, although their movement does not entirely cease. These groups constitute early blood islands. They are at first not surrounded by endothelial cells but later become enclosed or taken into the ends of the incipient vessels as briefly described above.

When the circulation first begins on the yolk-sac of a normal embryo a great many of these islands are present, but are more or less isolated or out of the channels through which the fluid is

flowing. The taking up of the islands by the circulation is most interesting to observe. At first those cells near the tail of the embryo, which are enclosed by endothelium, are taken. A few of the corpuscles are shaken by the current and these strike against the other members of the group until all become loosened and move slightly to and fro; finally one or two are suddenly washed away, then others follow—few at first, until the entire group loses its stand and is swept away by the current. One and then another of the islands may be seen to perish in this manner before the irresistible force of the tiny stream.

Yet even after the yolk circulation is fairly well established a number of islands of the round cells may still exist unsurrounded by the endothelial vessels. Figure 32 shows such a case. A well established current flows through the vessel to the right, while to the left is an incipient vessel not yet connected with the current. In the center of the figure is a group of corpuscles below a huge black chromatophore. These round cells constitute a blood island still unenclosed by vessel endothelium; in the course of a few hours, however, they too will become enclosed by a vessel and subsequently be included among the circulating blood cells.

The early erythroblasts which are in this manner included within the circulation assume a circular outline as they float in the current. In the previous paper, figures 31 and 32 of cross sections through the intermediate cell mass, and figures 34 and 35 of cells in a yolk island, all from a young 72 hour embryo, illustrate the definite circular outline of these cells. In life they may be carefully observed in the small vessels where a single cell passes with difficulty, and are here seen to be globular in shape and to retain their slight amoeboid movement. The form of the early erythroblast is readily changeable for the first one or two days after entering the current. After two or three days, that is, in embryos five or six days old, the cells in the blood current assume a typical erythrocyte appearance, becoming elongate and elliptical in shape when seen from one position, while they are thin in profile view. They are now ellipsoidal nucleated red blood corpuscles. At about the time they begin

to change from the globular to the elipsoidal shape, the accumulation of haemoglobin takes place and the cells begin to show a typical straw color.

It may then actually be seen in the living that the early or primitive erythroblast is really a more or less globular amoeboid cell without haemoglobin and resembling more closely a lymphocyte or early leucocyte than a fully formed erythrocyte. This is probably true of the early stages in many cases of cytomorphosis, yet the globular amoeboid cells of the yolk islands are not indifferent 'primitive blood cells' in the sense Maximow ('09) has concluded, but they are definitely future erythrocytes.

This point is established by a study of these cell groups in the normal as well as in embryos without a circulation of the blood. In the latter individuals the islands arise by the formation of local aggregations of the early wandering cells in an exactly similar manner to that described for the normal embryo. In fact, the observer cannot distinguish between the two specimens in many cases, yet one fails to establish a circulation and the islands are thus enabled to retain their positions on the yolk-sac.

The constituent cells of such a permanent island may be observed from time to time or continuously, and will be found to pass through changes exactly identical with those which take place in the island cells that become swept into the blood stream of the normal embryo. They are for a few days globular in shape, but capable of slightly changing their form and sending out short pseudopod-like projections. When the embryos are five or six days old the cells in these islands then become flattened ellipsoidal corpuscles and attain a haemoglobin content exactly as in the normal embryo. The blood islands now appear bright red in color and are quite conspicuous on the yolk-sac where they permanently remain. The globular colorless cells are thus seen to differentiate directly into the typical ictheoid erythrocyte.

From a study of the living embryos alone one could not, of course, be certain that all of the cells of these islands had differentiated into red blood corpuscles. However in the previous study of the non-circulating embryos, I have examined a large number of yolk islands completely and have never seen any

type of blood cells other than erythrocytes in such a position. The same is true of the cellular products of the intermediate cell mass; in hundreds of sections studied with the oil immersion not one case has been found of a lymphocyte or leucocyte arising from a cell of the original intermediate cell mass. It is thus concluded that this mass is the red-blood anlage of the teleost and the wandering cells of the yolk-sac which are very probably derived from the same mesodermal stem that forms the intermediate cell mass are likewise a portion of the red blood cell anlage.

The embryos that fail to develop a circulation are most important material for the study of these questions of relationship among the different types of blood cells. The observations on the living show definitely the qualities of the early blood forming mesenchymal cell in its assumption of the globular slowly wandering type, which would fully satisfy the descriptions of Maximow's 'primitive blood cell' as being closer in appearance to a lymphocyte than to a red corpuscle. When one follows these supposedly indifferent primitive blood cells which are claimed to possess the power of differentiating into either a leucocyte or an erythroblast, he invariably observes them to differentiate only into erythrocytes. Although all cells of the early islands are distinctly visible, they are not mixed in type, but are of one class. A long study of these early islands in sections also fails to reveal any other than red blood cells. The leucocytes are not very numerous in the blood, yet they should be seen if present in these islands, as they are found in other positions and are well represented in the blood of the adult Fundulus.

The further history of the cells in the stagnant masses on the yolk-sac of an embryo in which the blood has never circulated is instructive in several ways. In the first place, all of these islands seem to become surrounded by endothelium, yet the endothelial arrangement cannot be completely traced in every case and it rarely extends much beyond the island mass so far as one can observe in life.

Pigment cells are irregularly scattered in the neighborhood of the islands, as they are throughout the yolk region. The

chromatophores do not, however, assume any arrangement with reference to the islands of cells and as mentioned above they retain their original condition as separate cells instead of fusing into syncytial masses, as is the case in specimens with a free blood circulation.

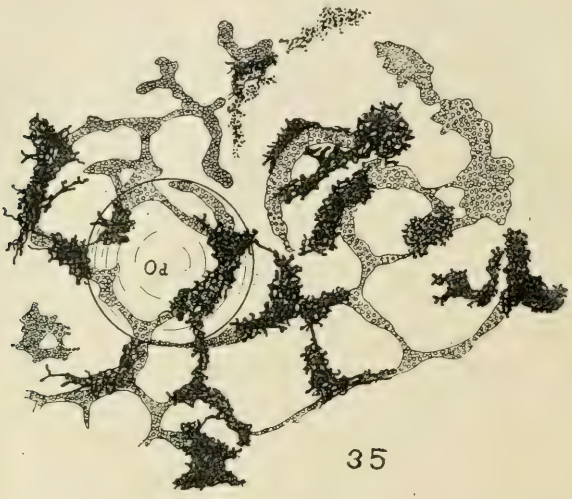


Fig. 35 The arrangement of red blood corpuscles in more or less connected endothelial sacs on the ventro-lateral surface of the yolk in an embryo 14 days old that had never had a circulation of fluid in its vessels. All of these blood cells wandered away from the caudal body regions of the embryo during early stages. The chromatophores are irregularly scattered among the old blood-islands. *Od*, oil drop.

Figure 35 illustrates a group of blood masses on the yolk-sac of an embryo 14 days old. In this specimen there had been absolutely no circulation or movement of the blood fluids within the vessels *at any time*. This is most important to know in the case of all specimens without a circulation at the period they chance to be examined. I shall return to this point below. The cell groups in the specimen without a circulation are arranged somewhat like a vascular net in the region illustrated by figure 35, yet they present a deadly still appearance as contrasted with the lively movement of the corpuscles within the yolk vessels of a normal individual. The erythrocytes forming these islands

are brilliantly red in color and their shape and size are apparently normal.

The blood corpuscles are thus found to differentiate in a typical fashion and to retain their haemoglobin reaction for a long period without having circulated in the vessels. The function of an erythrocyte would thus seem to be entirely independent of its circulation so far as its capacity to form oxyhaemoglobin goes. These cells are also able to accumulate oxygen within the intermediate cell mass in its central position in the embryonic body. The embryo from which figure 35 was taken had lived 14 days without its blood having circulated, which is about the period required for the young fish to hatch and become free swimming.

In older embryos the erythrocytes begin to degenerate and in many they lose their red color, the haemoglobin probably breaking down. The islands on the yolk then become pale in color and finally almost white, as if the cells were dead. The color of the blood cells seems to fade within the embryo earlier than on the yolk-sac as a rule, probably due to the better chances of obtaining oxygen on the thin yolk-sac than in the thicker embryonic body. The non-circulating specimens often continue to live for a long time even after the blood cells have lost their color. Some such specimens may exist for more than 40 days, which is a very long time considering that the normal embryo may hatch when from 11 to 20 days old. The specimens without a circulation are always weak and delayed in development and of course never succeed in hatching from the egg membrane.

In a study of the embryos treated with weak alcohol solutions, one very frequently finds cases in which the circulation of the blood may start almost normally and finally stop permanently, although the embryo continues to live. Other embryos may fail to establish a circulation at the proper time and yet may develop a freely flowing circulation of their blood some days later. Again, an embryo may have a fairly normal circulation and for some reason lose it for a few hours, or for one or two days, and then regain it, at first slowly and finally in a fairly strong fashion.

All three of these phenomena have also been observed in eggs developing in ordinary sea-water when they were not properly

separated so as to allow free respiration. The egg membrane is covered with long hair-like filaments which become entangled with those of neighboring eggs and in this way masses become closely packed. The central eggs of such a mass develop in a poor atmosphere and go more slowly than their neighbors on the outside of the mass. These centrally located eggs show many abnormal and arrested conditions of much the same type as may be obtained by treating the eggs with various injurious solutions.

The changeable states in the circulation offer many pitfalls for one attempting to determine the sites of origin of blood cells in the non-circulating embryos. Old embryos are seen in which there are beautiful blood islands on the yolk-sac and great clots of blood in the head or other unusual position. The heart is very frequently completely loaded with corpuscles, and yet there is not the slightest movement of the blood cells or any sign of a circulation at this time. The heart itself may be pulsating feebly or even practically stopped.

Another source of blood movement which is slight, yet to be guarded against, is that due to the muscular twitching of the embryo's body. This movement may frequently serve to push cells from the intermediate cell mass out on to the yolk-sac, but usually by way of the vessels. These dangers are to be taken seriously in experiments of this kind. Since one is able to be *absolutely certain* that the blood never circulates in a great number of embryos, only such embryos should be considered in a study of blood origin. During a study of this exact problem now extending over four spawning seasons, I have seen blood in almost every conceivable position in embryos without a circulation at the time of the observation. The accumulation of blood is more frequent in certain positions and regions than in others. The venous end of the heart is a most common place for a clot, the sides of the head, the large vessels of the yolk just lateral to the body, and various places on the anterior and lateral yolk surfaces.

When, however, the experimenter collects a number of embryos that have really never experienced the slightest flow of their blood, the case is very definite. No blood clots ever occur in regions other

than the 'intermediate cell mass,' within the embryo, and the islands on the caudal and ventral yolk surfaces which have been formed as described by the early wandering cells that migrate away from the caudal region of the embryo. All embryos whose history for lack of circulation throughout their existence is actually known show the blood pattern most consistently, there being of course a certain amount of variation in the extent and position of the yolk-islands but not enough in any case to confuse the problem.

These observations may readily be made by any observer, but can only be made in a reliable fashion with the high power microscope and strong condensers so that the light may be sufficiently regulated to observe the most transparent cells. The movements and differentiation of these cells should be carefully followed through every step in a number of cases, in order to fully appreciate the significance of their position and behavior.

The cells may be seen even with an ordinary binocular microscope to some extent, but the arrangements for light regulation and the magnification are insufficient for determining the important details. After the red blood cells have formed, they are readily located even with a low power yet such an examination could only determine their places of origin provided the embryo has been carefully watched with a high power magnification to make certain that it has had no blood flow.

The condition of the yolk-sac mesenchyme must be fully understood and must always be considered in interpreting the origin of blood-islands and clots. For example, clots seen at the venous end of the heart or on the extreme anterior surface of the yolk must be most cautiously considered, remembering the scarcity or even absence of the wandering mesenchyme in these regions. Clots in such places probably always result from a partial circulation of short duration and there is abundant evidence to support such a view.

Although the future red blood cells migrate upon the yolk in their early mesenchymal stages, after they once group themselves and differentiate into erythrocytes their powers of wandering become very much limited if they exist at all. I have never

seen anything to indicate that a fully formed erythrocyte was capable of automatic migration. Yolk-sac blood islands of all ages have been examined in great numbers, but never has an erythrocyte appeared wandering away from such an island into neighboring regions. This fact is most important in the study of the blood-islands in the non-circulating specimens.

When the slightest flow does exist for any length of time, there is a definite tendency, as mentioned above, for the blood to accumulate in certain sinuses and vessels. The positions of accumulation vary somewhat with the stages at which the circulation ceased, as well as the manner of stoppage of the flow, whether it was gradual or sudden.

When the circulation stops during early stages, there is a great accumulation or massing of the blood over almost the entire ventral surface of the yolk. In other words, there is a hemorrhage or bleeding into these spaces or vessels until no more blood is left in other regions of the embryo, the heart gradually becomes empty of corpuscles and no longer passes them along. The packing of the yolk vessels probably clogs or blocks the circulation so that it ceases. Again, the circulation may stop more suddenly and the venous end of the heart or the entire heart may be seen packed with corpuscles while the vessels immediately entering and leaving it are comparatively or entirely empty. In older embryos there is the tendency to accumulate red cells in the vessels of the head so that brilliant red clots are frequently seen in these positions.

In all cases it is interesting in these individuals in which the circulation has ceased at one or another period in development, and doubtless for different reasons in different specimens, to observe the way in which the blood sooner or later accumulates in one or another vascular space and does not remain uniformly distributed throughout the vascular system. Only when the heart is suddenly stopped and the blood quickly fixed by some strong killing fluid does one get a good pattern of the vascular system loaded with corpuscles throughout most of its extent. In rare cases, three during the present summer in some hundreds of embryos examined, will a specimen without a circulation at

the time observed show almost all of its vessels loaded with blood cells, and this is probably due to a slowing down gradually of the circulation on account of the heart itself which finally stops with the vessels in a balanced state.

The study of the yolk-sac in the living embryo enables one to observe every phase in the development of the red blood corpuscles from the early time when they wander as amoeboid mesenchyme cells to collect into groups of globular cells with short processes, the 'primitive blood cells' of descriptive histologists, to be later surrounded by vascular endothelium, and then to change from the globular wandering cells into the flattened ellipsoidal erythrocyte loaded with haemoglobin, and finally freely floating in the current of the blood stream. The fully formed corpuscles apparently become incapable of independently migrating even when not carried by the circulation.

DISCUSSION AND CONCLUSIONS

In the previous paper on the origin of blood and endothelium, a somewhat full discussion of the problems of blood formation in the teleosts and other vertebrates was entered into. A consideration of the questions of origin and development of vascular endothelium was also undertaken in the light of the results there presented and the more recent general literature bearing on this subject. The experimental results then contributed seemed in the light of the past literature to render highly probable, if not to actually prove, the polyphyletic origin of the various types of blood cells, as opposed to the now extremely improbable monophyletic theory of origin of blood cells and vascular endothelium. For a general consideration, the reader is referred back to these discussions.

A number of particularly significant points are brought out in the present study of the living normal and experimental embryos which bear directly on several of the past theories and speculations regarding the origin of vessels and blood. Only these special points will be briefly considered and analyzed at this time.

In the first place, the writer cannot resist the impulse to highly recommend that all students of haematogenesis and vascular origin spend some time at least in a study of living mesenchymal cells and their cytomorphosis. Such a study will soon convince one of the great disadvantages under which an investigator labors in attempting to solve the origin of blood from observations on dead material in serial sections. The problem becomes so simplified and devoid of laborious uninstructive technique that it seems almost superficial. One may learn as much from the living yolk-sac in an hour of careful study as in almost a week's perusal of sections. Most important is the fact that certain things may actually be seen to occur that sections could scarcely stimulate the mind to imagine. The only disadvantage is that the worker may be led to wonder whether so apparently simple a problem is actually of scientific importance. Fortunately this mental state is soon passed over on realizing the necessary care and precaution which must be taken in following the movement and changes in the living cells.

Each cell must be recognized as a living complex and the observer will realize the importance as well as the difficulties of thoroughly understanding and interpreting correctly its manifold changes and behavior. Material which to some extent allows such a study is often available. The Fundulus yolk-sac, however, is exceptionally adapted to this study on account of the beautiful simplicity of its structure, as well as the remarkable clearness with which each cell may be observed.

An investigation of the Fundulus yolk-sac readily supplies a crucial answer to the old question regarding the relation of the blood vessel lumen to other body cavities and spaces. Ryder ('84) was right in describing the blastocoel of the bony-fish as remaining an extensive cavity for some time. This is the space between the ectoderm and yolk and is identical and continuous with the cavity which arises very early beneath the blastoderm and above the yolk periblast. Agassiz and Whitman ('84), as well as Ryder ('84), Wilson ('90), and others, have identified this correctly as the cleavage cavity, the blastocoel. Later in development, the blastocoel extends over the yolk, forming the

space into which we have seen the free mesenchyme cells wander, and finally within this space groups of these cells form the yolk-sac vessels.

Wenckebach ('86) described very clearly the origin of vessels from the free mesenchyme within the segmentation cavity. My study of a somewhat similar yolk-sac confirms the main points brought out by Wenckebach and all serve as crucial facts in support of the early theory advanced by Bütschli ('82) in his "Die phylogenetischen Herleitung des Blutgefässapparates der Metazoen." Bütschli held that in the Metazoa the lumen of the blood vascular system was derived from the blastocoel. Later, Hubrecht ('86) supported the same standpoint from his studies on Nemertines. Hubrecht also found wandering cells playing an important rôle. Ziegler ('87) gives a most careful analysis of the continuity of the vascular lumen with the blastocoel in his studies on the development of the bony-fish.

The foregoing description and figures of the origin of vessels on the yolk-sac of Fundulus leaves no doubt that the vascular lumen in these animals, coenogenetically at any rate, is continuous with the blastocoel or primary body cavity and is in no way related to the coelom.

Almost twenty years ago, Felix ('97) advanced the opposing theory that the vascular lumen was really a localized or separate part of the secondary body cavity, or true coelom. The many decided objections to this theory from the standpoint of comparative anatomy, the presence of blood vessels before the acquisition of a true coelom in the animal kindgom, and the numerous embryological contradictions in its path were pointed out in the discussion of this matter in the previous paper.

Very recently Bremer ('14) has advocated the theory of the origin of vessels as parts separated from the coelomic cavity, or strands of cells from the coelomic epithelium. In the first place, the material on which his investigation was based, early human embryos, will scarcely permit such generalizations. At least more suitable material could be found for the analysis of this problem. Further than this, his consideration of the questions involved does not lead one to form a definite idea of

the exact direction he considers his evidence to lead. He credits Bütschli ('82) with having originated the coelom theory that accords, so he thinks, with his evidence. This is entirely incorrect, at Bütschli's theory is exactly on the other side. The morphology of the yolk-sac of the chick, sheep and numerous other animals, as the literature of the subject readily shows, is entirely out of accord with such speculations. The yolk-sac of the bony-fish shows this view to be really impossible and there should be no longer any doubt that vessels arise from loose and wandering mesenchymal cells in many animal species, and certainly not from ingrowths from the coelomic epithelium in any species.

The formation of vessels on the yolk-sac of the teleost further limits the generalization of the origin of larger vessels from capillary nets. Thoma ('93) in his masterly study of the vasculogenesis of the yolk-sac of the bird, held that "the first vascular spaces, the rudimentary capillaries, were formed by the secretory activity of the cells forming their wall." These capillaries formed an extensive net and the arteries and veins arose secondarily and differentiated from the capillaries on account of the flow of blood set in motion by the beat of the heart. The anlage of the vascular system was the capillary.

These principles of Thoma are not, however, applicable to the development of vessels in the embryonic bony-fish. The aorta arises as one or two vessels independent of any flow of blood or the existence of a capillary net. The first vessels on the teleostean yolk-sac are the large vitelline veins, as described by Wenckebach, and the median vitelline vein or the net of vessels in its place. These large important channels arise entirely independently and separated from the capillary net if such exists at the time. They also develop entirely independently of the blood flow, and not as a result of the pressure due to the heart beat. The capillaries and other vessels in many cases arise separately or away from these primary vessels and finally connect with them in a way similar to the connection formed between the Randvene and the venous end of the heart. Other capillaries and small vessels arise as buds or sprouts from the first formed veins on the yolk-sac.

More recently Evans ('09) with a very efficient and delicate method of injection has shown many of the larger intra-embryonic vessels of the chick embryo to develop from a foregoing capillary network. He found the same principles of development that Thoma had observed on the yolk-sac to hold for the development of certain vessels within the embryo. These principles of vascular development Evans thought applied to vertebrates generally, but such is certainly not the case, the large vessels of the teleost embryo arise directly from associated mesenchymal cells and are not preceded by a capillary net.

His evidence was derived from injected vessels and could not justify the statement, p. 512, of "The presence *always* in the embryo of a united vascular system"—"a single branched endothelial tree." Such a united vascular system is rather late in its establishment in the fish embryo and there is no "single branched endothelial tree" present when the first blood vessels are formed. These facts may readily be demonstrated on the living embryo by direct observation.

The vessels of the yolk-sac and several of the larger vessels within the body of the teleostean embryo form independently of any foregoing capillary network. *In the teleost, then, the anlage of the vascular system is not the capillary but the mesenchymal cells which directly give rise to the chief arteries and veins, as well as to numerous groups of isolated capillaries.* Other small vessels and capillaries grow as branches or sprouts from the arteries and veins.

Thoma advanced three laws for the formation and growth of vessels. The first law was considered the most important, but rather destructive evidence is thrown against it by the present study. The law may be stated as follows: "The increase in the size of the lumen of the vessel, or what is the same thing, the increase in the surface of the vessel wall, depends upon the rate of the blood current." The vessel increases in size when the rate is exceeded, becomes smaller when the rate is slowed, and *disappears when the flow is finally arrested.* Thoma ('96) states: "This law which brings the growth of the surface of the vessel into dependence upon the rate of the flow of the blood is, I con-

sider, the first and most important histo-mechanical principle which determines the state of the lumen of the vessel under physiological and pathological conditions."

Thoma again states this principle thus: "In development the vessels in which the blood stagnates degenerate, and in those in which the rapidity is too great the lumen in enlarged."

No one could fail to admire the splendid manner in which Thoma attacked the problem of vasculogenesis in the yolk-sac of the bird, or the ingenious way in which he attempted to analyze the problem and deduce his three laws of histo-mechanical processes. Yet the "First and most important histo-mechanical principle" does not apply to the development of vessels in Fundulus embryos where there is no circulation of the blood. *Many vessels grow in size or "what is the same thing, show an increase in the surface of the vessel wall" without any "rate of the blood current."* The aorta in old embryos that never had their blood to circulate and in which the heart is actually a solid string of tissue, grows and attains a well developed lumen and a wall lined with endothelium and surrounded by concentric fibers of connective tissue as is shown in figure 49 in the previous paper, drawn from such a specimen. This vessel is very slow to degenerate, in fact, it shows no sign of degeneration and actually persists as long as the embryo is able to exist without a circulation, for 30 days or more. Vessels also develop upon the yolk-sac without ever having a fluid to circulate through their lumen. *Other vessels are developed around the blood cells of the intermediate cell mass and the yolk-sac islands and in such vessels 'the blood stagnates' from the first yet the vessels degenerate very slowly, in some cases scarcely at all.*

In still other cases the blood may have circulated for a while and then stopped for some time, but the vessels do not degenerate as is proven by the fact that the circulation through them may again be resumed. Such a sequence of events may be occasionally observed in the experimental embryos. *The function of the vessel as a blood conductor, therefore, seems in these embryos of Fundulus*, both the early normal and those without a circulation of the blood, *to have little if anything to do with its early development and not much effect on its ability to survive.*

On the other hand, when Thoma has a straight normal case, the lumen may readily be seen to increase in size with the rate of flow. Yet in the entire absence of this action the vessel is still capable of increasing in size and it becomes questionable whether the rate of flow is ever an actual cause of size increase beyond mechanical stretching.

These facts are most significant in a consideration of the influence of function on growth and development, auto-differentiation. Here it is seen that the structure both grows and develops in entire absence of its function. In normal cases the function of the vessel as a blood conductor exerts more likely a physical rather than a biological effect on development.

Thus the development of blood vessels on the yolk-sac of the living Fundulus embryo proves that the capillaries are not universally the anlage of the arteries and veins, but that these larger vessels may arise directly from wandering mesenchyme cells. Such arteries and veins may grow and persist without a circulation of the blood through their lumen and even though stagnant masses of corpuscles may crowd the vessel cavity.

The development of vessels in Fundulus also directly disproves the claims made by Sobotta ('02) that the vessels in the teleost grow over the yolk entirely as branches from those near the embryo and without the wandering cells taking part. This assumption is probably due to the difficulty of estimating the part played by wandering cells from a study of serial sections. From a study of the living yolk-sac there is no question of the major part played by the wandering cells in the origin and formation of vessels on the yolk.

Sobotta also advances the opinion that the entire yolk vessels may sprout from the heart. This is much of the same nature as the ingrowth or parablast theory of His ('75), and is obviously defeated by the same array of facts which long ago relegated the parablast theory to a place of mere historic interest, in spite of the fact that it is so often revived for literary reasons.

Finally, we may consider the study of the developmental products of the early wandering mesenchymal cells on the yolk-sac of the Fundulus embryo as a problem of cell lineage carried

to its ultimate end. The primordial mesoderm cell or cells carry within their bodies all the potentialities of the mesoderm and may give rise to a series of cells which are capable of developing muscle, cartilage, bone, connective tissue proper, blood cells, vessels, etc. Yet after a few cell generations the individuals in the series derived from these early cells containing all the mesodermal potentialities no doubt become somewhat limited as to their potentialities. In a certain generation there may be definite cells more or less generally distributed which possess the capacity to give rise to muscle cells, but to no other type of mesodermal tissues. Still later in development these cells may come to be even more limited in their developmental capacities and thus have the power to produce only a certain type of muscle cell and no other type.

Collections of such cells would then be designated embryologically as the anlage of striated muscle, smooth muscle, or heart muscle as the case might be Yet it is not to be forgotten that at this stage there might be really no means of distinguishing between the several different types of mesodermal cells.

Limitization of potentialities in the individual cells has apparently reached a comparable stage just about the time when the mesenchymal cells begin to wander upon the yolk-sac of Fundulus. We have seen these cells as they wander out and have noted how very soon they may be separated into four distinctly different types, and following the development and behavior of these types it has seemed evident that they are entirely separate and do not intergrade or transmutate. The black chromatophore does not change its nature or divide off other cells which become different in type from the parent cell. Neither do the endothelial cells lining the vessel walls change into chromatophores or into erythroblasts, or vice versa.

From all the observations on these yolk-sacs we must conclude that the four types of cells described above have developed from four different anlagen, although these anlagen were not necessarily localized groups of cells, but were diffusely scattered mesenchymal cells capable of developing into a definite product, either normal or abnormal, depending upon the nature of the

developmental environment. Therefore, the four distinct mesenchymal anlagen each give rise to a perfectly typical and distinct cell type although all develop in, as far as one can judge, an identical environment, the cavity of the yolk-sac between the ectoderm and the periblastic syncytium. The differences among the four cell types produced are from the standpoint of our present knowledge in all probability due to the potential differences among the apparently similar mesenchymal cells from which they arose. The four types including endothelial cells and erythrocytes we must consider from an embryological standpoint as arising from different mesenchymal anlagen.

SUMMARY

The yolk-sac of the teleost egg is a most beautiful object for observing the movements and migrations of cells in the developing embryo. Such a yolk-sac has only one really definite continuous membranous cell layer, the ectoderm; a true endodermal layer is absent, though a superficial syncytium, the periblast, fuses with the actual yolk surface. The mesodermal layer is represented by numerous separate wandering mesenchymal cells. These freely wandering mesenchymal cells may be clearly observed through the perfectly transparent ectoderm as they move over the surface of the periblast.

The present contribution attempts to give a full account of the movements of the mesenchyme cells and their manner of development and differentiation in the yolk-sac. Observations have been made on the normal embryos from the earliest stages at which the mesenchyme wanders out upon the yolk up to the late embryo in which a complex vitelline circulation is fully established, and all of the products of the yolk mesenchyme completely differentiated. The study has been greatly facilitated by a comparison of the normal embryos with specimens in which the circulation of the blood was experimentally prevented from taking place. In such specimens the cells on the yolk-sac never became confused or contaminated with other cellular elements introduced by the circulating blood. The

wandering cells may thus be completely followed through all stages in their isolated position.

The behavior of the migrating cells impresses one with the very important rôle of such elements in the formation of tissues and organs, particularly the blood vessels and certain blood cells. The observer is also struck by the fact that such phenomena are extremely difficult if not actually impossible to interpret from a mere study of dead specimens cut in serial sections. Of course the study of sections greatly aids the observations on the living, and but for the fact of a long acquaintance with the Fundulus yolk-sac in sections, the writer would have found it much more difficult to identify many of the cells in life.

The results of this investigation of wandering mesenchymal cells may be summarized as follows:

1. The wandering cells begin to migrate away from the embryonic shield or line of the embryonic body at an early period, when the embryo is about 40 hours old, the germ ring having almost completely passed over the yolk sphere to enclose its vegetal pole. The cells migrate away chiefly from the caudal end of the embryo, only a few wandering out from the head region. The regions of the yolk-sac thus suggest an area opaca about the tail end and an area pallucida around the neighborhood of the head.

All of the cells wander into the so-called subgerminal cavity, the space Wilson ('90) and others consider a late stage of the segmentation cavity, between the yolk-sac ectoderm and the periblast syncytium.

When the cells first appear they are all closely similar in shape and about the same size. Very soon, however, they begin to exhibit certain differences. Many become elongate spindle cells with delicate filamentous processes, sometimes producing a stellate appearance. Others are more amoeboid in shape with conical pseudopod-like processes which are constantly being thrown out at one place and withdrawn at another. Still a third class of cells appear somewhat later than the other two; these are more circular in outline with short thick pseudopods and are more slowly moving.

The movements of these extremely numerous cells and their changes of position may be readily followed with a high magnification. In embryos of about 60 hours, still some time before the heart begins to beat or the blood to flow, four clearly distinct types of cells may be recognized among these originally similar mesenchymal cells, and the further history of the four types has been completely traced.

2. The amoeboid cells with conical pseudopod-like processes shortly after 60 hours begin to show an accumulation of pigment granules within their cytoplasm. Just at this time they are seen to be of two distinct varieties, one depositing a black and the other a brownish red pigment.

The black chromatophore increases rapidly in size and by the end of the third day becomes an enormous amoeboid body wandering over the yolk. These cells are attracted to the walls of blood vessels and plasma filled spaces, such as the pericardial cavity becomes in specimens without a blood circulation. When the embryo is five days old the chromatophores are abundantly arranged along the walls of the vitelline vessels, but the pigmented cells are distinctly separate. After this time neighboring cells begin to fuse along their adjacent borders and large pigment syncytia are formed which completely surround and ensheath the vessels. A single syncytium is often of considerable extent, as shown in figure 15.

The brown chromatophores have a somewhat different history. They never become so massive as the black, and their processes are more delicate and graceful in appearance. Yet these cells also attain a large size and in embryos of 72 hours are scattered over the entire yolk-surface. After the third day when the blood begins to flow in the yolk vessels, the brown chromatophores likewise become attracted to the vessel wall. These exquisitely branched cells apply themselves to the wall of the vessel and may often completely surround it, as shown in figure 17. This type of chromatophore, however, always maintains its cellular individuality and never fuses with other cells to form a syncytium as is the case with the black type.

The function of the chromatophores on the yolk-sac is most difficult to decide, but one thing is certain, they never become

changed into any type of blood cell. The brown chromatophore in early stages may accidentally reach the blood current; it then becomes spherical and may be readily observed for a long time on account of its huge size as compared with the blood cells. It never, however, changes in type.

In specimens without a circulation of the blood both types of chromatophores arise in a normal manner and differentiate normally. Their arrangement along the vessel walls fails to occur and they remain scattered over the yolk or collected about the plasma filled spaces. The heart in such embryos is sheathed with pigment, while the normal heart never has a chromatophore on it.

3. The elongate spindle cells with their delicate filamentous processes are small in comparison with the two chromatophore types. These spindle cells retain in general their original appearance, but their behavior is most important. In embryos of about 48 hours such cells aggregate into certain rather definite groups; later, these groups become more linear in shape and finally these lines of cells arrange themselves so as to form tubular vessels. Several of the larger vessels arise independently upon the yolk, and certain ones of them later become connected with the venous end of the heart, while in all cases capillary nets which also arise independently become connected with the larger vessels. These processes may actually be followed through every step in the living yolk-sac.

The wall of the early vessels is very irregular with spaces existing between the component cells. Corpuscles are often caught in these spaces or entangled in the filamentous processes of the endothelial cells. Such conditions in sections would appear as though the corpuscles actually formed a part of the endothelial wall and might incorrectly be interpreted as endothelial cells changing into blood cells. Nothing has been seen in the living embryos to indicate that an endothelial cell has the power to produce a blood cell or to change into a blood cell of any type, but much has been seen to the contrary.

The generalization particularly made by Thoma ('93) that larger vessels arise from a net-work of capillaries is not true for the large vitelline vessels on the fish yolk-sac. It is also found

in the specimens without a circulation of the blood that the vessels arise and increase in size and persist for a long time without ever experiencing any effect of the blood current upon their walls. In many embryos the circulation after having begun may stop for a time and then later be reestablished, the vessels having persisted in a normal condition. Thoma's so-called laws of vessel formation are, therefore, rudely violated by the development of the vascular system in these embryos.

The vessels arising from independent mesenchymal cells in the space of the blastocoel in the teleost yolk-sac entirely overthrow any notion that vessels arise ontogenetically as portions of the coelomic epithelium. The vascular lumen is originally continuous with the primary body cavity, the segmentation cavity, and never with the secondary body cavity or coelomic cavity.

4. The fourth class of cells wander out from the embryonic body somewhat later than the three former types. These are small globular cells with short pseudopod-like processes. They move very slowly, but finally collect into groups on the posterior and ventral regions of the yolk-sphere.

The round cells wander away only from the caudal region of the embryo and probably are derived from the so-called intermediate cell mass which is the anlage of the red blood corpuscles in the fish embryo.

The groups of round cells are slow in their differentiation but just before the circulation of the blood begins, they are seen to be circular erythroblasts. The observer may follow the disappearance of the islands of cells one by one as they are enclosed by the vessels and swept into the circulating stream. About the fifth day these circular erythroblasts become flattened ellipsoidal erythrocytes filled with haemoglobin, the typical red blood corpuscle. The complete change from wandering more or less spherical mesenchymal cells into typical haemoglobin bearing corpuscles may be followed in the living yolk-sac.

In several instances the entire body proper of the embryo failed to develop or else degenerated very early, yet the yolk-sac formed or persisted with numerous blood islands fully differentiated.

The embryos in which there has been no circulation of the blood form the blood islands from the wandering cells on the yolk-sac, and the constituent elements of these islands differentiate perfectly and may maintain their red color for many days. Yet they never leave the locality in which they have differentiated. The fully formed red blood corpuscles have little if any power of migrating. When the observer can be positive that the blood has never circulated, and this requires very consistent watching, the islands of the yolk are always limited to certain regions and never occur so far anteriorly on the ventral surface of the yolk as to reach the venous end of the heart.

5. On the yolk-sac of Fundulus embryos one thus finds four distinctly different products differentiating from the apparently similar wandering mesenchymal cells. The environment in which the four types differentiate is identical as far as is possible to determine, and the only explanation of their various modes of differentiation is that the original mesenchymal cells that wandered out were already of four potentially different classes. These differences in potentiality within the early cells gave rise to the four different directions of cytomorphosis in one and the same environment. The four resulting types of cells are then in an embryological sense derived from different mesenchymal anlagen.

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